The Royal Veterinary and Agricultural University

Salmonella Dublin in Dairy Cattle

Use of diagnostic tests for investigation of risk factors and infection dynamics

Ph.D. Thesis

Liza Rosenbaum Nielsen
Department of Animal Science and Animal Health
June 2003

Supervisors:
Annette Kjær Ersbøll, Associate Professor, Cand. Scient, Ph.D., Department of Animal Science and Animal Health, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark

Peter Lind, Professor, Senior Scientist, D.Sc., Department of Immunology and Biochemistry, Danish Veterinary Institute, Copenhagen, Denmark

Assessment committee:
Hans Houe, Professor, DVM, Ph.D., D.V.Sc., Department of Animal Science and Animal Health, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark

Ian Gardner, Professor, Ph.D., M.P.V.M., B.V.Sc., Department of Medicine and Epidemiology, University of California, Davis, USA

Herman Barkema, Associate Professor, DVM, Ph.D., Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, Canada

***Salmonella* Dublin in Dairy Cattle**
Use of diagnostic tests for investigation of risk factors and infection dynamics

ISBN: 87-7611-013-3
Printed by Samfundslitteratur Grafik, Frederiksberg, Denmark
Cover design: Pil Molbech Bøggild

Salmonella Dublin in dairy cattle

Use of diagnostic tests for investigation of risk factors and infection dynamics

Contents

Preface

The present thesis was written as part of my PhD study at the Royal Veterinary and Agricultural University (KVL) in Denmark. The PhD project was part of "the Integrated Cattle Health and Milk Quality Project" (also known as the "Kongeå-project"), initiated in 1997 and funded by the Danish Dairy Board. The "Kongeå-project" formed a fundamental structure behind my PhD project and opened great opportunities for collaboration and collection of samples and data material by offering massive support through funding, administration and expertise. I am very grateful to have had the opportunity to be part of the Kongeå-project, and I would like to thank the entire salmonella project group and other helpful people from the Danish Dairy Board/Danish Cattle Federation (Astrid Mikél Jensen, Jørgen Nielsen, Marianne Skovbogaard, Erik Rattenborg, Rikke Thind), the Danish Cattle Health Laboratory/Steins Laboratory in Ladelund (Viggo Bitsch, Anna Bodil Christoffersen, Britta Nylin), the Danish Veterinary Institute (Dorte Lau Baggesen, Nils Feld, Annette Nygaard Jensen, Lars Erik Larsen), the Danish Agricultural Advisory Center and KVL for their support during the last 3½ years. Also, thank you to the group of people behind the National Surveillance Program for *Salmonella* Dublin for supporting the outbreak investigations and for constructive work with the "tool-box" for intervention strategies (including Gert Berthelsen, Jacob Roland Pedersen and many of you already mentioned above).

Another great opportunity for me was to be part of the Research School for Animal Health and Production (RAPH), where I was offered very relevant courses expanding my horizon, and where I was able to do both social and scientific networking with other PhD students as well as faculty from many scientific fields and parts of the world. Thanks for all the support and challenges. Special thanks to enthusiastic Pia Haubro Andersen, former leader of RAPH, for insisting that researchers need to know more than just how to do research.

This PhD thesis was truly made through blood, sweat and tears – but also jokes, wonders and pleasant surprises. Without the many hours of hard work performed by all the milk quality advisors (in particular Jan Nelson, Niels K. Sørensen, Lars Mortensen and Bent Jensen) from the Danish Dairy Board who collected thousands of samples from the dairy herds involved in my project, without the laboratory staff at the Veterinary Department at Steins Lab (Anne Marie Sørensen, Sanne Prüsse, Anna Helene Lindved, Merete Højberg, Gunhild H. Smidt, Lone M. Jørgensen, Inga Filtenborg, Tina Demolade, Tina B. Asmussen and everybody who helped in the salmonella lab) and at DVI (Gitte Sørensen and Anders Hay Sørensen), and without the commitment of the open-minded farmers of the "Kongeå-project" and the local veterinarians (Rødding Dyrehospital, Arnum-Gram Dyrlæger I/S, Toftlund Dyrlæger, Løgumkloster Dyreklinik, Vamdrup Dyreklinik, Ribe-Kalvslund Dyrlæger, Per H. Dinesen and Peter Oluf Krag), the "CASADY" database that forms the

basis of my work would never have been as extensive and useful as it is today. Thanks! Your work and commitment will be appreciated for many years to come.

It may be that I would have performed this work anyway, but probably at a slower pace had it not been for Birgitte Langvad, who continued to request I got moving! (not a bad thing at all). It was fun working with you, Birgitte.

In the Fall of 2002, I spent 2½ months at Department of Population Medicine and Diagnostic Investigation at Cornell University, where I had the great privilege of working with two very committed epidemiologists, Ynte Schukken and Yrjö Gröhn. It was rewarding to spend time exploring my data and performing in-depth epidemiological analyses. Your comments and great discussions that were continued after my stay were greatly appreciated, and I hope we will be able to do more work together in the future.

The value of good supervisors during a PhD project cannot be overestimated. Annette Kjær Ersbøll and Peter Lind have been patient and supportive throughout the whole process, both regarding scientific work and practical matters. Thank you both!

It is hard to say who made the most difference during my PhD study, because many people did, but I owe the following people a lot for helping me see things in new perspectives, or for helping me out when I was stuck:
My office mate for the last 3 years Søren Saxmose Nielsen for great discussions and informative illustrations on our black board, help with all those little things that continue to tease when using SAS and Excel, good gossip, proof readings, patiently listening to my complaints etc. etc.!! Line Nissen and Hanne Skovsgaard Sørensen for hours of late night work with too little sleep out in the darkest and coldest parts of Jutland during the calving project – and always smiling, too. You really made that project work! Carsten Enevoldsen for plenty of good ideas and encouragement. My “I am watching you and I will nag you if you get sloppy”-colleague Nils Toft. The epi-group at IHH including Jeanne Oakman who herself makes every working day more pleasant. Lis Alban for cheering me up when I did not see a way out - but also for keeping my feet on the ground. Per Bundgaard Larsen for providing antibiotic treatment data. Frank Hansen for helping me visualize my data, which made me change my approach. Tina Birk Jensen for fruitful discussions and collaboration and for taking interest in my work. Pil Molbech Bøggild for artistic contribution to the cover, and last but not least my amazing husband, Peter Bøggild, for all the things you did that nobody else could have done for me!!

Copenhagen, June 2003
Liza Rosenbaum Nielsen

Summary in English

This thesis is about *Salmonella* Dublin in dairy cattle with particular focus on interpretation of diagnostic test results from traditionally used tests such as ELISA and faecal culture tests. It also examines risk factors for becoming clinically ill during an outbreak, and for becoming a persistent carrier of *Salmonella* Dublin upon infection in outbreak herds and endemically infected herds.

Salmonella Dublin is a bacteria that is host-adapted to cattle and which causes both economical and welfare losses in the cattle industry. Approximately 25% Danish dairy herds are considered infected with *Salmonella* Dublin according to the Danish National Surveillance Program for *Salmonella* Dublin. Unlike most other types of salmonella bacteria, *Salmonella* Dublin has a tendency to reside in the herds for years or decades, mainly due to its ability to produce persistently infected carriers that shed bacteria to the environment either continuously or periodically. In order to control the infection in such endemically infected herds, it is necessary to cull the carriers and prevent production of new carriers, in addition to general preventive measures taken against infectious diseases. Therefore, the focus of this thesis is to increase the understanding of the diagnostic tests available for detection of *Salmonella* Dublin infected animals and to provide new knowledge about factors influencing the risk that animals become carriers.

The thesis is composed of seven chapters, a list of references and two appendices. Chapter 1 gives the background for the work presented, objectives of the thesis and an outline. Chapter 2 provides a general introduction to *Salmonella* Dublin and a literature review of subjects of relevance for the studies and manuscripts presented in the thesis. It also gives a short introduction to "the Danish National Surveillance Program for *Salmonella* Dublin" that was initiated in October 2002. Chapter 3 provides a thorough description of the materials and methods used throughout the thesis including the framework of "the Integrated Cattle Health and Milk Quality Project" that the presented work was part of, the sampling activities and laboratory procedures providing salmonella data for the resulting database, called "CASADY". The structure and application of this database is described.

Chapter 4 concerns diagnostic test validity and interpretation. Besides an introduction to the validity parameters (in particular test sensitivity and specificity) and to the methods for estimating these parameters, this chapter provides results from Study 1 "Relative validity of faecal pools compared to individual samples", which shows that pooling of faecal samples

using 5 g faecal matter from each of 5 animals in one pool with follow-up at individual level if the pool is positive for *Salmonella* Dublin (also called the pool-first method), reduces the sensitivity of the faecal culture test by 57%. Study 2 "Validity estimation of the individual milk ELISA" shows that the milk ELISA can be used for detection of infected animals if the imperfect sensitivity and specificity is taken into account. The study provides estimates of these parameters at different cut-off values and the results can therefore be used in intervention trials and surveillance programs. The results from these studies are discussed in relation to the results from Manuscript 1, "Age stratified validation of an indirect *Salmonella* Dublin serum ELISA test for individual diagnosis in cattle", and Manuscript 2, "Validation of an indirect serum ELISA and a bacteriological faecal culture test for diagnosis of *Salmonella* serotype Dublin in cattle using latent class models". These manuscripts show that the serum ELISA has the best overall performance in calves and young stock 100-299 days of age when compared to younger calves and cattle 300 days or older. These two manuscripts provide estimates for sensitivity and specificity at different cut-off values of the serum ELISA in these three age groups of cattle, using both classic and latent class methods for estimation. The latent class method also provides a rough estimate of the sensitivity of the pool-first faecal culture method. Finally, factors influencing bulk tank milk ELISA response against *Salmonella* Dublin is discussed and related to the results from Manuscript 3, "What determines the variation in the bulk tank milk response against *Salmonella* Dublin in dairy herds?". It is shown that factors related to the mean individual cow ELISA response are related to the bulk tank milk ELISA response, and that knowledge of the infection status of the herd is not necessary to predict the outcome of the bulk tank milk response. The bulk tank milk ELISA response was mainly related to the cows and much less related to the infection status among young stock and calves.

Chapter 5 provides an introduction to known risk factors for *Salmonella* Dublin infection in cattle. Results from two smaller studies are provided, Study 3, "Risk factors for salmonellosis in cows during an outbreak of *Salmonella* Dublin" and Study 4, "Risk factors for becoming a carrier cow after an acute outbreak of *Salmonella* Dublin". These studies show that the main risk factor for a cow to become clinically ill from *Salmonella* Dublin during an outbreak is stage of lactation, with periparturient cows being at highest risk. Study 4 shows that cows that were clinically ill during the outbreak had the highest risk of becoming carriers. A short summary of results from Manuscript 4, "*Salmonella* Dublin infection in cattle: Risk factors for becoming a carrier" is included. The conclusions from Manuscript 4 were that animals close to calving as well as heifers had a higher risk of becoming carriers upon

infection than cows in mid to late lactation. Herd prevalence of infected animals was a protective factor, suggesting that low exposure to *Salmonella* Dublin was associated with a higher risk of becoming a carrier upon infection in endemically infected herds. Also, there appeared to be a weak association between season and the risk of becoming a carrier with the highest risk being in later Winter and Spring.

Chapter 6 contains a discussion of the results and conclusions from the four studies and the four manuscripts. It provides an evaluation of data quality, an overall discussion and perspectives of the work in the present thesis. It provides suggestions for future work including mathematical modelling of within-herd infection dynamics of *Salmonella* Dublin based on the conclusions of the presented work. Finally, practical recommendations based on the conclusions of the present thesis are given. The four manuscripts can be found in Chapter 7.

Sammendrag på dansk (Summary in Danish)

Denne ph.d. afhandling handler om *Salmonella* Dublin hos malkekvæg, og fokuserer på fortolkning af diagnostiske testresultater fra traditionelt anvendte test så som ELISA og bakteriologisk dyrkning af fæcesprøver. Ydermere studeres risikofaktorer for at udvikle klinisk sygdom og for at blive persisterende smittebærer af *Salmonella* Dublin efter infektion i udbrudsbesætninger og endemisk smittede malkekvægsbesætninger. *Salmonella* Dublin er en bakterie, der er værtsadapteret til kvæg, og den giver både økonomiske og velfærdstab i kvægbranchen i dag. Ifølge det danske nationale overvågningsprogram for *Salmonella* Dublin menes ca. 25% danske malkekvægbesætninger at være smittede med *Salmonella* Dublin. I modsætning til de fleste andre typer af salmonellabakterier har *Salmonella* Dublin en tendens til at medføre persisterende infektion hos nogle dyr, som enten kontinuerligt eller lejlighedsvis udskiller bakterier til omgivelserne via gødningen og dermed bidrager til, at bakterien forbliver i besætningen i årevis. For at bekæmpe og kontrollere infektionen i endemisk smittede besætninger, er det udover almindelige præventive foranstaltninger mod infektiøse sygdomme nødvendigt at udsætte dyr med persisterende *Salmonella* Dublin infektion og at forhindre, at der bliver produceret nye persistent inficerede dyr. Derfor er der i denne afhandling fokuseret på at øge forståelsen af anvendte diagnostiske tests til udpegning af *Salmonella* Dublin-inficerede dyr og på at tilvejebringe ny viden om faktorer, der påvirker risikoen for at kvæg udvikler sig til persistent inficerede dyr.

Afhandlingen består af syv kapitler, en referenceliste og to bilag. Kapitel 1 giver baggrunden for de præsenterede studier, specifikke formål og en oversigt over afhandlingens indhold. Kapitel 2 giver en generel introduktion til *Salmonella* Dublin og en opsummering af litteraturen indenfor emner af relevans for studierne og manuskripterne i afhandlingen. En kort introduktion til det nationale overvågningsprogram af *Salmonella* Dublin, som blev startet i Danmark i oktober 2002, kan også findes i dette kapitel. Kapitel 3 indeholder en grunding gennemgang af materialer, der bliver anvendt igennem hele afhandlingen, inklusiv et skitsering af ”Kongeå-projektet”, som lå til grund for det arbejde, der præsenteres her. Desuden beskrives prøveindsamlingerne og laboratoriemetoderne, der har tilført salmonelladata til den resulterende database ”CASADY”. Strukturen og anvendelsen af denne database illustreres.

Kapitel 4 omhandler validiteten og fortolkningen af diagnostiske tests. Udover en introduktion til validitetsparametre (hovedsagelig testenes sensitivitet og specificitet) og metoder til

at estimere disse parametre viser kapitlet resultater fra Studie nr. 1, "Relative validity of faecal pools compared to individual samples". Studiet viser, at pooling af gødningsprøver med 5 g gødningsmateriale fra hvert af 5 dyr med opfølgende dyrkning af gødning fra individer i dyrkningspositive pools (pool-først metoden), reducerer sensitiviteten af dyrkningstesten med ca. 57%. Studie nr. 2, "Validity estimation of the individual milk ELISA", viser at individmælke-ELISA kan bruges til at detektere *Salmonella* Dublin-inficerede dyr, hvis der tages forbehold for at sensitiviteten og specificiteten ikke er perfekte. Der opgives estimater for disse parametre ved forskellige cut-off værdier og resultaterne kan derfor bruges i interventionsforsøg og overvågningsprogrammer. Resultaterne fra Studie 1 og 2 diskuteres i relation til resultaterne fra Manuskript 1, "Age stratified validation of an indirect *Salmonella* Dublin serum ELISA test for individual diagnosis in cattle", og Manuskript 2, "Validation of an indirect serum ELISA and a bacteriological faecal culture test for diagnosis of *Salmonella* serotype Dublin in cattle using latent class models". Disse manuskripter viser, at serum ELISA overordnet set har den bedste validitet, når den bruges på kalve og ungdyr mellem 100 og 299 dage gamle, i forhold til når den bruges på yngre kalve eller ældre dyr. Estimater for serum ELISAs sensitivitet og specificitet angives for forskellige grænseværdier i de tre aldersgrupper, når der blev brugt en klassisk estimeringsmetoden og når der blev brugt en latent-klasse-metode. Til slut i Kapitel 4 diskuteres faktorer, der har indflydelse på tankmælksmålinger af antistoffer mod *Salmonella* Dublin målt med en mælke-ELISA, og der relateres til resultaterne fra Manuskript 3, "What determines the variation in the bulk tank milk response against *Salmonella* Dublin in dairy herds?". Det vises, at faktorer, der hænger tæt sammen med det gennemsnitlige ELISA-respons i lakterende køer har stor betydning for tankmælksresponset, og at viden om besætningens infektionsstatus ikke er nødvendig for at kunne forudsige tankmælks-ELISA-responset i malkekvægsbesætninger. Tankmælks-ELISA-responset er hovedsageligt relateret til køerne og i mindre grad til infektionsstatus blandt ungdyr og kalve.

I Kapitel 5 gives en introduktion til kendte risikofaktorer for *Salmonella* Dublin infektion hos kvæg. Derefter præsenteres resultaterne fra to mindre risikofaktorstudier, Studie nr. 3, "Risk factors for salmonellosis in cows during an outbreak of *Salmonella* Dublin" og Studie nr. 4, "Risk factors for becoming a carrier cow after an acute outbreak of *Salmonella* Dublin". Disse studier viser, at en vigtig risikofaktor for sygdom forårsaget af *Salmonella* Dublin hos køer under et udbrud er laktationsstadiet. Køer, der befinder sig i peripartum, har signifikant højest risiko for at blive syge af *Salmonella* Dublin. Studie nr. 4 viser, at køer der blev klinisk syge under et udbrud er dem, der har højest risiko for efterfølgende at

udvikle sig til persistent inficerede dyr. Et kort referat af resultaterne fra Manuskript 4, "*Salmonella* Dublin infection in cattle: Risk factors for becoming a carrier" er inkluderet i kapitlet. Konklusionerne fra Manuskript 4 er, at dyr der inficeres med *Salmonella* Dublin tæt på kælvningstidspunkt, eller mens de er kvier (fra løbning til første kælvning) har højere risiko for at blive persistent inficerede, end dyr der inficeres fra midten af til sent i laktationen. Besætnings-prævalensen af *Salmonella* Dublin-inficerede dyr var en beskyttende faktor, hvilket kunne betyde, at de dyr der smittes med *Salmonella* Dublin trods lavt smittepres har større risiko for at blive persistent inficerede, end dyr der smittes under middel til højt smittepres i endemisk smittede besætninger. Der var også en svag sammenhæng mellem årstid og risikoen for at udvikle sig til persistent inficerede. Den højeste risiko var sent på vinteren og i foråret.

Kapitel 6 indeholder en diskussion af resultater og konklusioner fra de fire studier og de fire manuskripter. Der foretages en evaluering af datakvalitet, en overordnet diskussion og perspektivering af det præsenterede stof. Forslag til fremtidige studier, inklusiv udvikling af en matematisk model for *Salmonella* Dublin-infektionsdynamik indenfor besætninger og anbefalinger til praktiske tiltag baseret på afhandlingens konklusioner er samlet her. De fire manuskripter er samlet i Kapitel 7.

Chapter 1. Introduction

1.1 Background

Salmonella enterica subsp. *enterica* serovar Dublin (*Salmonella* Dublin) is a cause of acute and subclinical disease in cattle. It can cause disease in cattle of all ages, though the most commonly clinically affected group is calves aged 2 weeks to 3 months[137,142]. *Salmonella* Dublin is also a serious zoonosis[30,100]. Official registrations report 20-50 clinical human salmonellosis cases in Denmark every year due to *Salmonella* Dublin[109]. This is a smaller number of cases than human salmonellosis cases caused by other types of salmonella bacteria. However, the invasive nature of *Salmonella* Dublin causes a significantly higher mortality rate in patients hospitalized with salmonellosis due to *Salmonella* Dublin than in patients hospitalised with salmonellosis due to other types of salmonella bacteria, such as *Salmonella* Typhimurium or *Salmonella* Enteritidis[45].

Salmonella Dublin also interacts differently with its primary host, cattle, than other salmonella bacteria do. It is common to see haematogenic and lymphatic spread of the bacteria to the internal organs, joints and the central nervous system, where the bacteria may produce a typical picture of a septicemic infection with placentitis, pleuritis, peritonitis, nephritis, arthritis, osteomyelitis and meningoencephalitis[6,95]. These manifestations occur in addition to endotoxic fever and enteritis followed by dehydration and systemic shock, which are the most common symptoms in salmonellosis caused by most other salmonella bacteria[138]. In the cattle industry, *Salmonella* Dublin causes economic losses in the form of death among calves and young animals, abortions and reproductive disorders among adult cattle, extra labour and increased veterinary expenses[47,89,124]. Thus, controlling *Salmonella* Dublin infections in cattle herds can provide economic, health and welfare benefits in the cattle industry, and may reduce the zoonotic risk.

An important feature of *Salmonella* Dublin is the ability of the bacteria to produce persistent carriers[29,40,92]. The bacteria spreads through the lymphatic fluids and blood to lymph nodes and internal organs, where it may reach an inactive state so that the animals appear healthy. These animals are often called latent carriers. The bacteria may become reactivated in such latent carriers, which then start to excrete large numbers of bacteria and thus become infectious to other animals and people around them. This is likely to be one of the main reasons why *Salmonella* Dublin often establishes itself as an endemic infection in the herds it enters[138].

The fact that *Salmonella* Dublin infection may take very different courses in the individual animals after the initial transmission makes it challenging to interpret and use results from traditional and available diagnostic tests such as bacteriological culture and enzyme-linked immunosorbent assays (ELISAs). Validity of several diagnostic tests for detection of infected animals have shown to be highly dependent on the actual infection stage and age of the animal[21,46,51,91,93,104,105,108,121]. Although most researchers agree that ELISAs are useful, varying success has been reported from attempts to use ELISAs in control or eradication of *Salmonella* Dublin from infected herds[54,56,81]. A bulk tank milk ELISA has been developed for detection of immunoglobulins directed against *Salmonella* Dublin[50,132]. In the Netherlands, this test is used for voluntary certification of uninfected dairy herds based on bulk tank milk testing 3 times a year[118,120]. In Denmark, the test is used in a national surveillance program that classifies all cattle herds into three infection levels based on ELISA response in bulk tank milk samples collected every quarter of the year in dairy herds, and three blood samples collected yearly from non-milk producing herds[8].

Thus, considerable interest has focused on this infection over the last couple of decades and has caused a high demand for knowledge about how to interpret test results, in particular bulk tank milk ELISA responses in relation to the dynamics and levels of the infection within the herds, and individual ELISA response in serum and milk for detection of carrier animals. However, in order to study the infection dynamics within herds and risk factors affecting the spread of the infection, more knowledge about the validity of tests for individual diagnosis is required, and approaches for using these tests over time and in combination need to be explored.

1.2 Aim and specific objectives

The aim of this PhD thesis is to provide new knowledge about interpretation and validity of available diagnostic tests (i.e. bacteriological culture methods and ELISAs), and to use these tests to gain new knowledge about within-herd infection dynamics of *Salmonella* Dublin. Based on better understanding of test validity and interpretation, the aim is to examine pieces of the puzzle of within-herd dynamics of *Salmonella* Dublin through risk factor studies for clinical salmonellosis and production of persistent carriers during *Salmonella* Dublin outbreaks and endemic infection in dairy herds. Finally, the aim is to quantify factors contributing to variation in bulk tank milk ELISA response in dairy herds.

The specific objectives of this thesis are:

- To critically evaluate the validity of diagnostic tests used for *Salmonella* Dublin infection in:
    a. Bacteriological culture of faecal samples
    b. Bacteriological culture of pooled faecal samples
    c. Serum ELISA
    d. Individual milk ELISA
    e. Bulk tank milk ELISA

- To study risk factors related to infection dynamic aspects of *Salmonella* Dublin

The tests are evaluated using both classic and latent class analyses.

The hypotheses regarding evaluation of diagnostic tests were:

1. The sensitivity of faecal bacteriological culture of *Salmonella* Dublin in cattle is age-dependent and can be estimated using latent class test validation
2. Pooling of faecal samples reduces the sensitivity of the bacteriological faecal culture method for detection of *Salmonella* Dublin bacteria
3. The validity of the indirect serum ELISA for detection of *Salmonella* Dublin infection in cattle is age dependent
4. The sensitivity of individual ELISAs are better than the sensitivity of faecal culture methods for detection of *Salmonella* Dublin infection in cattle
5. Unbiased sensitivity and specificity estimates of individual ELISAs can be obtained using latent class analysis as opposed to using classic test validation methods.
6. The bulk tank milk *Salmonella* Dublin ELISA response reflects the level of infection particularly in the lactating cows, and the presence of *Salmonella* Dublin infection in a herd
7. The variation in bulk tank milk ELISA response can be explained by factors related to the lactating cows

Risk factors for becoming clinically ill during an outbreak of *Salmonella* Dublin in a dairy herd and risk factors for becoming a carrier during an outbreak and under endemic situations in dairy herds were analysed based on diagnostic test results and registrations in 12 dairy herds with known *Salmonella* Dublin infection.

The hypotheses regarding risk factors were:

8. The risk of salmonellosis in cows during an outbreak of *Salmonella* Dublin is influenced by the lactation stage, parity and level of milk production at time of exposure.
9. The risk of becoming a carrier after an outbreak of *Salmonella* Dublin in a dairy herd is influenced by host and management related factors at time of infection
10. Reduced immune capacity of young calves and animals under increased stress load such as around the time of calving may increase the risk of animals becoming carriers instead of recovering, when they are infected with *Salmonella* Dublin.

1.3 Outline of the thesis

The thesis contains seven chapters. Chapter 2 provides the relevant scientific background for the work behind this thesis with regard to *Salmonella* Dublin pathogenesis, immune response and transmission of the infection. It also gives a short introduction to the Danish surveillance program for *Salmonella* Dublin. Chapter 3 contains a thorough description of the data collection activities, laboratory procedures and resulting database on which the analytic work of this thesis is built. Results are presented in Chapters 4 and 5 in four studies and in Chapter 7 in four manuscripts. The subject of Chapter 4 is diagnostic procedures, and the chapter reports results of test validations and investigations of how to interpret the laboratory test results, including descriptive and analytic examinations of the variation in individual and bulk tank milk response over time in dairy herds. Chapter 5 contains a review of risk factors for *Salmonella* Dublin infection reported in the literature and provides results from analyses of risk factors for clinical disease and carrier development. Chapter 6 discusses and links the results to provide overall conclusions and perspectives of the presented work, and finally Chapter 7 contains the four manuscripts.

Chapter 2. *Salmonella* Dublin infection in cattle

2.1 Historical perspective

In 2003, approximately 2600 serotypes of salmonella bacteria have been named and characterized, and the number is constantly growing thanks to new molecular techniques that facilitate differentiation between the different types of salmonella bacteria. The two most frequently found salmonella serotypes in cattle are *Salmonella* Dublin and *Salmonella* Typhimurium[29,137]. The first written reports about salmonellosis in cattle were probably from year 1865 when "calf paratyphoid" outbreaks were described in Germany, the Netherlands and Denmark. Exactly which bacteria and which serotype caused these outbreaks is unknown[137]. In 1891 C.O. Jensen was the first to describe the bacteria responsible for these outbreaks and reproduce the disease by experimental infection in calves[58]. He named it *Bacillus paracoli,* and found that it was likely to be a very pathogenic and invasive variant of another rod-shaped enteric bacteria, which was found in healthy animals and only produced disease in immunosuppressed animals. In the beginning of the 1900s, reports of outbreaks in both adult cattle and young animals from both the USA and Europe described the bacteria in more detail, though the nomenclature was somewhat confusing. This was improved in the extensive work by F. Kauffmann and B. White, which lead to description of a great number of salmonella serotypes often named from the geographical location, where the first strain was isolated[39]. In 1926 J.W. Bigger reported the isolation of a similar bacteria from a human case in Dublin, Ireland[88]. It was initially thought to be a *Salmonella* Enteritidis, but in 1939 it was determined to be an individual serotype and was named after the place of isolation. The serotypes are differentiated based on the three major antigenic determinants: the flagellar H antigen, the somatic O antigen and the Vi antigen. The O- and H-antigens are the most important for classification of *Salmonella* Dublin.

One of the first reports about outbreaks of *Salmonella* Dublin, after it was acknowledged as a salmonella serotype, were from calves and young stock from 2 weeks to 12 months of age. The outbreaks occurred in calves purchased for experimental purposes at the University of Cambridge, UK, and the infection showed very high morbidity (up to 90%) and high mortality (17-80%). Since then, multiple reports on bovine salmonellosis due to *Salmonella* Dublin – both outbreaks and endemic disease in both calves and adult cattle have appeared in the literature[48,70,79,115,123,132,139]. Varying morbidity and mortality rates have been reported. Also clinical symptoms and pathological findings have varied between cases.

Some of the first reports documenting *Salmonella* Dublin as a zoonosis were from 1938 and 1947 in the UK, where milk from either clinically ill cows or apparently healthy cows excreting the bacteria were found to be the sources of the infections. After pasteurisation became commonly used, the number of human cases have decreased dramatically. Outbreaks of human *Salmonella* Dublin cases have recently been traced to both unpasteurised cheese, milk and other dairy products[30,134]. Also beef meat is a known source of human *Salmonella* Dublin infections[9].

2.2 Pathogenesis

Salmonella Dublin is often considered host adapted to cattle[102,139], which means that cattle is the most common host of *Salmonella* Dublin. Other species, including pigs[63], sheep[24,71], human beings[30,45], rats[41], chinchillas[128] and fowl[78] have also been reported to become clinically ill from *Salmonella* Dublin infection. The mechanisms of the host adaptation are poorly understood, but most likely relate to genetic traits of both the bacteria and the host.

The pathogenesis of *Salmonella* Dublin can be seen as a series of more or less overlapping steps: uptake of bacteria and passage to the small intestines, colonization of the intestinal lumen, invasion of the intestinal epithelial cells, uptake by macrophages in the underlying lymphoidal tissues, dissemination via the lymph fluid to other organs rich in reticuloendothelial tissues (e.g. lymph nodes, liver, spleen, bone marrow) and the circulating blood. Regulation of the pathogenesis relates to both host factors and bacterial factors, and much research has been and is currently being performed in this field to develop a better understanding of the importance of different factors and how they influence each other[64,99]. It is, however, still an area of great controversy. Thus, only aspects of importance for the rest of the work presented in the present thesis are covered here.

2.2.1 Route of infection

The most frequent route of infection is ingestion of the bacteria through contaminated feed, water or milk. Other less common routes of infection may include the conjunctiva and airways[80,127], ascending infection through the teat canal to the udder[107] or through the genital tract from where it may disseminate to the rest of the organism via the lymph fluid or blood

and usually also lead to faecal excretion of bacteria. Transplacental infection from mother to foetus may be possible as will be described in further detail in section 2.2.6.

2.2.2 Colonization and invasion

Salmonella bacteria are normally inhibited by the high concentrations of volatile fatty acids (VFAs) in the rumen and the normal pH below 7 in the rumen[18,69], acidic secretions of the abomasum (pH below 4.8)[95], the normal peristalsis of the gut, which prevents adhesion to the epithelial cells and the normal competing microflora of the intestines. Salmonella bacteria have developed mechanisms to survive and cope with these inhibiting factors, but the normal inhibition of salmonella bacteria is primarily disrupted in the rumen and small intestine when (i) starvation or reduced feed intake occur, such as during transportation or when the animal is ill for other reasons[18,73], (ii) the feeding strategy leads to an increased pH in the abomasum, most often seen in young calves during the milk feeding period, (iii) antibiotic treatment kills the normal competing microflora of the intestine, and (iv) changed motility of the gut occurs[11,80].

The infectious dose may be sufficiently high even under normal conditions to allow large numbers of *Salmonella* Dublin bacteria to reach the lower small intestine, where colonization takes place. Usually, peroral infection doses of 10^6 or more leads to clinical symptoms, bacteraemia and faecal excretion, though the severity of the symptoms, length and degree of excretion vary considerably with age and prior immunity[80,96,101,111]. Direct intraduodenal inoculation of 2×10^4 or more bacteria were found to invariably lead to severe disease unless the animal had some evidence of specific immunity to the infection prior to inoculation[80].

The bacteria adhere to and invade intestinal cells in the mucosa mainly associated with the Peyer's patches in the terminal jejunum and ileum through the columnar enterocytes and specialized microfold enterocytes (M cells). The enterocytes present receptors to the antigens of the bacteria and through a process of membrane ruffling passes the salmonella bacteria to the lymphatic tissues underneath. Once the bacteria have crossed the intestinal epithelium they enter macrophages in the underlying lymphoid tissue from where they are drained to the local lymph nodes, which are important barriers for further dissemination. If this barrier is overcome, the bacteria reach the reticuloendothelial tissue containing organs while surviving and replicating inside the macrophages[99,101]. A large number of genetic

traits in the bacteria determine the degree of invasiveness, adhesion to phagocytes, survival and proliferation in the macrophages, and there is still much research being performed to examine exactly which of these traits are important or necessary, and which genes work in conjunction to determine the outcome of the infection[11,28,64,129,130].

2.2.3 Virulence

The virulence of *Salmonella* Dublin is a measure of the severity of disease caused by the bacteria in the host. The virulence of *Salmonella* Dublin is probably influenced by Salmonella plasmid virulence (Spv) genes of this serotype as it is the case with other host adapted salmonella types, such as *Salmonella* Cholerasuis, *Salmonella* Gallinarum-pullorum and *Salmonella* Abortusovis. The Spv genes promote intracellular proliferation in intestinal tissues and at extraintestinal sites such as the liver and spleen in cattle[65,125]. Spv genes are also found in some isolates of *Salmonella* Typhimurium and other types of salmonellae, but only a proportion of isolates carry the virulence plasmids. The virulence plasmid genes are not necessary for disease to occur, but according to Libby et. al (1997)[65], the symptoms in experimentally infected calves are much more severe and mortality higher, when infected with *Salmonella* Dublin wild-type strains carrying the Spv genes, than in strains without Spv genes. In-vitro proliferation was shown to be high in macrophages with *Salmonella* Dublin wild-type strains, whereas strains without the Spv genes had no proliferation of the bacteria in the infected macrophages.

A study of 135 *Salmonella* Dublin isolates from Danish cattle and humans in 1983-1987 showed that 99% of the strains carried at least one plasmid, and that the virulence plasmid profiles of *Salmonella* Dublin strains found in Denmark were very similar. Comparison of plasmid profiles between isolates from humans and cattle suggest that the same clones infect both cattle and humans in Denmark. A very frequently found plasmid in these strains was a 80 kb, serotype-specific and virulence-associated plasmid of *Salmonella* Dublin[86]. This finding suggests that most *Salmonella* Dublin strains in Denmark are virulent, and that plasmid profiling may be of limited value for epidemiological studies.

2.2.4 Lipopolysaccharides

An important property of salmonella bacteria is the part of the outer cell membrane consisting of lipopolysaccharide components (LPS). LPS is important for the pathogenesis. It is in direct contact with the environment and provides protection against the very different

environmental factors the bacteria meet (e.g. dryness of the air, the UV-rays of the sun, the acidity of the abomasum). LPS consists of three major parts; the Lipid-A, the core oligosaccharide and the serotype-specific O-antigens that protrude into the environment around the bacterial cell[98]. LPS is responsible for stimulation of the immune system. For instance, immuno-globulin A (IgA) directed against the O-antigens is part of the local immune defence in the gut and blocks mucosal invasion by binding to the antigen sites. Mouse models have shown that an *Lps* locus in the host governs the ability to respond to LPS. This may influence the host's susceptibility to salmonella infection[99]. The immune response to salmonella infection will be addressed in more detail in section 2.3.

Lipopolysaccharides are endotoxins that during infection contribute to vascular damage and thrombosis. They cause fever, disseminated intravascular coagulation, circulatory collapse and shock during salmonella infection[27]. LPS is practical for use in classification and diagnostic tests for salmonella bacteria. The O-antigens are, together with the flagellar H-antigens, essential for the serological differentiation between serotypes. *Salmonella* Typhimurium has O-antigens 1, 4, 5, 12 and *Salmonella* Dublin has O-antigen factors 1, 9 and 12. The common O-antigen factors 1 and 12 may cause some cross-reaction in O-antigen based serological tests. This will be illustrated further in Chapter 4. Some strains of *Salmonella* Dublin also possess the less common Vi antigen, which is a true capsular polysaccharide that is associated with virulence through inhibition of phagocytosis[98].

2.2.5 Pathology, clinical signs, morbidity and mortality

Experimental infection with *Salmonella* Dublin shows that the bacteria have special affinity for the columnar enterocytes of the terminal jejunum and ileum, the follicle-associated epithelium (FAE) over the Peyer's patches, and glandular tissues in the duodenum, tonsillar area in pharynx and the lungs in calves aged 6 to 28 weeks[101]. This study also showed that susceptibility and development of disease was both age and dose dependent with the most severe symptoms including death from septicemia and acute necrotizing panenteritis occurring within 24 hours in calves aged 6-7 weeks infected with a peroral dose of 2×10^9 bacteria. Mucoid diarrhoea, high fever, loss of appetite, weakness, discoloured mucous membranes and mucoid nasal discharge were the most prominent signs in calves aged 12-14 weeks infected with a peroral dose of 2×10^{10} bacteria. In the oldest age group of calves aged 25-28 weeks, the only symptom of infection was a slight increase in rectal temperature on days 2-3 after peroral infection with 1×10^{10} bacteria. *Salmonella* Dublin was found in

faecal samples from all infected animals. The immunohistochemical investigations after autopsy of the experimental animals showed presence of *Salmonella* Dublin throughout the intestines. The columnar enterocytes of the terminal jejunum and ileum were invaded and the villi were degenerated. The epithelial cells of the duodenal glands, bile ducts, gall bladder, retropharyngeal glands and abomasum contained large numbers of *Salmonella* Dublin, but only moderate signs of degeneration and invasion of inflammatory cells were seen. These findings were similar to another experimental study, where severe lesions in the lungs, heart and kidney were also reported[80].

In a field study performed in England in 1968-69 in 223 herds with clinical salmonellosis due to *Salmonella* Dublin, adult dysentery with low morbidity (few affected cows), but high mortality (close to 50%) was found in 41 herds. *Salmonella* Dublin related abortions with very few other clinical signs were found in 31 herds. Abortions were most frequent in the seventh month of pregnancy. Calf salmonellosis was found in 184 herds. Out of 145 calves diagnosed with *Salmonella* Dublin at post-mortem examination, the age varied from 3 days to 18 weeks (mean of 4.4 weeks), the morbidity was in the range of 26-37% and the mortality was 13-18% during the outbreaks[94]. With some variations in the age distribution of affected animals between outbreaks, these findings are fairly similar to other field studies from different parts of the world[70,123,138,139].

The variation of clinical appearances of salmonellosis caused by *Salmonella* Dublin can be summarised in a convenient set of categories in which a few to all of the symptoms may be seen simultaneously[90,95,137]:

- Peracute infection - quick death with very few clinical symptoms, septicaemia. Usually caused by very high doses or very virulent strains in fully susceptible young calves, but may also be seen in adult cows or heifers in the beginning of an outbreak of virulent *Salmonella* Dublin infection in a fully susceptible herd.

- Acute infection – fever, unthriftiness, depression and lack of appetite, pneumonia, (bloody) diarrhoea, (poly)arthritis and osteomyelitis leading to lameness and hot, swollen joints, meningoencephalitis leading to nervous symptoms in calves. Bloody diarrhoea, fever, depression, abortion, decreased milk production and lack of appetite in adult cows.

- Chronic infection – mostly seen in animals older than 6-8 weeks. Failure to thrive, bloody and loose stool, intestinal casts, slightly elevated temperature, scruffy hair coat and growth retardation.

Abortion may be seen in any stage of the infection, usually during the middle or last trimester of pregnancy[90] and is often the only symptom expressed by the aborting animal[48].

2.2.6 Pathogenesis of abortion

An experimental study illustrated the pathogenesis of abortion caused by *Salmonella* Dublin in heifers aged 27 to 44 months infected at the 180th day of pregnancy[43]. The conclusions from the study were that following intravenous inoculation of *Salmonella* Dublin bacteria, the infection spread to the spleen, liver, lung and associated lymph nodes within 2 days of inoculation. Often fever accompanied this dissemination of bacteria. The bacteria could be found in high numbers in the placentomes after 6-8 days, and just before abortion rapid multiplication of *Salmonella* Dublin occurred in the connective tissue of the cotyledons. It was suggested that the placental destruction during this multiplication of bacteria lead to hormonal changes, which initiated abortion. Another report of the same study showed that abortion follows a second period of pyrexia 5-11 days after inoculation depending on the infectious dose. Three aborting animals inoculated with high doses of bacteria (0.1–1.4 x 10^{10}) became severely ill[42]. A study of routine investigations of bovine abortions performed in 1970-1972 concluded that other clinical signs were rare in 111 abortion cases associated with *Salmonella* Dublin[47]. Together, these studies suggested that smaller doses of *Salmonella* Dublin may cause abortion, but usually abortion is only followed by other clinical signs if the aborting animal received a high dose of the infection. This was also supported by another field study showing very few other symptoms in 36 aborting cows with *Salmonella* Dublin recovered from the placenta[94]. If the heifer or cow does not abort and the foetus survives the transplacental infection, congenitally infected calves may be born. It still remains to be shown how often this occurs and what factors influence the birth of congenitally infected calves. Also, the importance of such congenitally infected calves with regard to transmission of the pathogen in the herd remains to be determined.

2.3 Immune response and excretion of *Salmonella* Dublin bacteria during infection

2.3.1 Basic concepts of the immune system

The immune system consists of two major parts: The non-specific (also called the innate) immunity and the specific (also called the acquired) immunity. The specific immune system is further divided into two compartments: the cell-mediated immunity and the humoral immunity. The different components of the immune system act in combination to remove invading organisms and substances that are foreign to the body. As illustrated in Figure 2.1, the non-specific immunity is the first host barrier that micro-organisms have to pass in order to infect an animal. It consist mainly of physical barriers and chemical components (e.g. intestinal motility, abomasal acidic secretions, complement pathways) and inflammatory cells circulating in the blood and lymph fluid (e.g. macrophages, neutrophils, natural killer cells) and their secreted cytokines[99]. The activity of macrophages and the activation of the complement pathways are enhanced by interaction with antibodies from the humoral immune system. This implies that the non-specific immunity may be strongly influenced by the specific immunity. As described below, the non-specific immunity also stimulates the specific immunity, and the three compartments work in unity to combat intruding organisms and foreign antigens.

Lymphocytes are the most dominant cell types of the specific immunity. The B cells originate from the bone marrow and are primarily located in the cortex of lymph nodes, Peyer's Patches and the spleen as naive B cells presenting antibody molecules (immunoglobulins) on their surfaces. All antibody molecules on a given B cell have the same antigenic specificity, and can interact directly with the antigen. Such interaction will initiate a primary immune response through an endocytic processing pathway that presents degraded antigen as peptide-major histocompatibility complex (MHC) II on the surface of the antigen presenting cells. T helper cells (T_h cells) recognise and bind to these complexes via their receptors. Clones of such a stimulated B cell will proliferate and differentiate into specific antibody producing plasma cells and memory B cells. Plasma cells can secrete more than 2000 antibody molecules per second and live for a few days. Since the process takes some time to initiate, the primary humoral immune response is delayed 5-7 days with regard to free specific immunoglobulins circulating in the blood. After the initial stimulus, antigen-specific memory B cells continue to circulate in the blood and lymph in higher numbers than the naive B cells. Thus, if the animal is again exposed to the antigen, the secondary

humoral immune response is both quicker (1-2 days) and 100-1000 fold higher than the primary immune response[36].

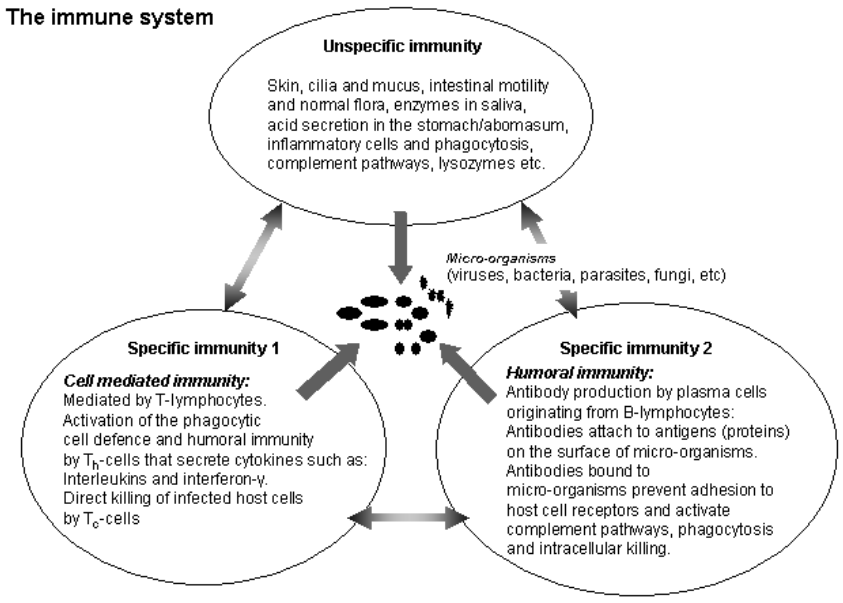


Figure 2.1 A schematic presentation of the different compartments of the immune system (modified from Houe et al. (2002)[52]).

T lymphocytes also originate from the bone marrow, but circulate in the blood and mature in lymphoid organs, such as the thymus. After maturation, the T cells express unique antigen-binding molecules, called T cell receptors, on their membranes. The T cell receptor can only recognise antigen that is bound to one of two classes of MHC molecules. MHC class I molecules are bound to most nucleated cells, whereas MHC class II molecules are expressed by antigen presenting cells (i.e. macrophages, dendritic cells and B cells). When the naive T cells meet antigens bound to MHC-molecules, they proliferate and differentiate into memory T cells and different types of effector T cells: the T helper (T_h) cells that display CD4 membrane glycoproteins, and the T cytotoxic (T_c) cells that display CD8 membrane glycoproteins on their surfaces. A CD4 expressing T_h cell that recognises an antigen-MHC class II molecule complex becomes activated and starts to excrete cytokines (e.g. interleukins, interferon-γ, transforming growth factor-β). The cytokines play an important role in

activating B cells, T_c cells and macrophages in the microenvironment around the cells excreting them[136]. The T_h cell response is an essential part of the immune system. Without it, very few antibodies are produced by B cells, and cytotoxic T_c cells are not produced. Also, so-called delayed type hypersensitivity (DTH) response that is important in combatting many intracellularly proliferating micro-organisms, such as salmonella bacteria, will not occur without cytokine stimulation from T_h cells. The pattern of excreted cytokines determines the type of elicited immune response with regard to isotype distribution of immunoglobulins (i.e. IgG1, IgG2, IgE, IgM etc). Under the influence of cytokines from the T_h cell response, CD8-coated T_c cells that recognise antigen-MHC class I molecule complexes become cytotoxic T lymphocytes that have direct eliminating activity on altered self-cells (e.g. virus-infected cells, tumor cells)[36].

2.3.2 Immune response to *Salmonella* Dublin infection

The innate immunity is important as the first barrier against invading salmonella bacteria. It mainly acts against colonization and invasion of the intestinal wall by salmonella bacteria. Outer membrane lipopolysaccharides (LPS) of enterobacteria impose a mixed set of beneficial and adverse reactions on the host related to the non-specific immunity. Binding of LPS to macrophage membranes sensitises these cells to the action of interferon-γ (IFN-γ), which is one of the inflammatory cytokines that control the early bacterial growth. Small amounts of LPS raise a beneficial inflammatory response, but large amounts of LPS can induce overproduction of cytokines, which results in tissue damage and endotoxic shock[76].

The acquired immune response acts together with the innate immune system components in protection against subsequent infection and to cure late and latent infection. The cell-mediated immunity acts by direct killing of the infectious agent, killing of infected cells or activation of phagocytic cell defences. The T_h1 response involves development of naive helper T cells into T_h1 cells by secretions of interleukin-12 by infected macrophages and IFN-γ by natural killer cells during the early phase of the infection. T_h1 cells activate the microbicidal properties of uninfected macrophages and induce some production of circulating antibodies that bind to and help eliminating extracellularly located bacteria[99]. A strong humoral immunity is induced by T_h2 lymphocytes and acts through production of antibodies against the major cell surface components and secretions of the bacteria (e.g. LPS, Vi polysaccharide, flagellae and other surface proteins).

Salmonella Dublin has developed an ability to survive and proliferate intracellularly in macrophages. One of the reasons may be LPSs, which protect the bacteria against lysozymes in the phagosomes of the macrophages. Furthermore, salmonella bacteria lack certain necessary proteins and receptors necessary for lysozymatic degradation of the bacteria in the macrophage phagosomes. In delayed type hypersensitivity, IFN-γ activates macrophages to kill pathogens more effectively. A 10-day delayed non-specific macrophage activity against intracellular pathogens, activated by IFN-γ and IL-2 released from sensitised T_h1 cells, was reported by Tizard (2000), p. 260[114]. This indicates the importance of the cell-mediated immunity against *Salmonella* Dublin. It also suggests why somewhat immunosuppressed animals may not be able to clear the infection effectively. A host gene named Nramp1 has been shown to control the resistance to intracellular organisms such as salmonella bacteria[28]. The gene is probably only expressed in macrophages[122]. While the cell mediated immune response is important for the susceptibility to infection and ability to eliminate the bacteria from the organism, it is not necessarily correlated to the level of circulating immunoglobulins[49].

Though, in general, the immunity of a foetus is much less developed than in mature cattle, different immune reactions to infections of the foetus may occur at different developmental stages dependent on the infectious organism in question. Exactly when the foetus will be able to produce immunoglobulins against salmonella bacteria is not known, but it is likely to be in the last half of the pregnancy. At birth, the foetus/calf is immunosuppressed due to increased foetal cortisol levels[10]. Further, cell-mediated immunity is deficient at birth. Therefore, calves are very susceptible to salmonella infection at this time. Around 2 weeks of age the cell-mediated immune capacity reaches levels similar to adult cattle. These mechanisms make the calves very dependent on antibodies, phagocytic cells and cytokines passively transferred from dam to calf through colostrum. More than 85% of the immunoglobulins in colostrum consist of IgG_1, and the IgG_1 concentration is 5-10 times higher than IgG_2 in colostrum. The half life of colostral-derived immunoglobulins in the neonate is between 11.5 and 16 days[10].

Adult cattle has four main isotypes of immunoglobulins: IgG_1, IgG_2, IgM and IgA. IgE, IgG_3 and IgG_4 can be found in lower concentrations. The half life of bovine immunoglobulins are approximately 10-18 days for IgG_1, 18-32 days for IgG_2, 4-4.8 days for IgM and 2.5-3.4 days for IgA[14]. The functions of the different immunoglobulins vary. IgG_1 is selectively transported by several types of cells, e.g. intestinal crypt cells and alveolar

epithelial cells in the mammary gland, leading to accumulation of IgG_1 in milk, in particular colostrum. The IgG_1 concentration in colostrum is 5-10 times higher (30-75 mg/ml) than the IgG_1 concentration in serum (6-15.1 mg/ml) of the cow[14]. Local production of immunoglobulins also occur. The concentration of IgG_1 measured in milk more than 14 days postpartum is in the range 0.33-1.2 mg/ml, whereas the IgG_2 concentration is 0.037-0.06 mg/ml). In serum the levels of IgG_1 and IgG_2 are similar (6.0-15.1 and 5.0-13.5 mg/ml, respectively).

Salmonella Dublin has been shown to survive in the mammary gland for more than a year despite high levels of specific anti-*Salmonella* Dublin IgG[107], which indicates that the humoral immunity is not sufficient to clear this type of infection. Except for the different immunity functions of immunoglobulins, they are also useful markers of infection, because agent specific immunoglobulins can be measured in serological tests, for instance enzyme-linked immunosorbent assays (ELISAs).

Robertson et al. (1982)[97] showed that 15 *Salmonella* Dublin infected calves aged 2-4 months that had faecal excretion at least three times during a period of 6-10 weeks previous to the study, had a significant increase in double skin fold thickness 48 hours after injection of crude extract from *Salmonella* Dublin and *Salmonella* Enteritis. The pathological changes seen in the biopsies from the skin swellings showed massive cellular infiltration of mononuclear cells typical of a delayed type of hypersensitivity suggesting a strong cell-mediated immunity was present in the infected calves compared to the uninfected control group. Humoral antibody titers were not correlated to the extent of skin swellings[97].

According to Chaturvedi and Sharma (1981)[19] cell mediated immunity is most important in protection of calves against *Salmonella* Dublin infection compared to humoral immunity. These authors found that calves with well-developed cell mediated immunity showed few and mild symptoms upon reinfection even with high infection doses, and that also excretion of bacteria were shorter than in control calves. Calves with high levels of immunoglobulins from passive transfer did not have the same degree of protection.

Other studies have shown that animals with some degree of humoral immunity develop less severe or no clinical symptoms and also excrete bacteria for a shorter period of time after inoculation of *Salmonella* Dublin bacteria in dosages causing typical symptoms in fully susceptible calves[40,80,111], but these studies did not consider the cell mediated immunity.

Age has proven important for the ability of the animal to produce immunoglobulins against *Salmonella* Dublin infection[21,110]. Calves below the age of approximately 11-12 weeks have a significantly reduced antibody response to LPS. This may be part of the reason why this group of cattle is more susceptible and often develop more severe disease when infected with *Salmonella* Dublin.

2.3.3 Transiently infected animals

Experimental studies have shown that excretion of bacteria occurs within 12-24 hours after inoculation, and that it is not necessarily related to disease. In one study excretion of *Salmonella* Dublin dropped to very low and intermittent levels after 15 days, and only few animals still excreted bacteria 45 days after the initial infection dose was given[96]. The IgM titer started to increase after approximately one week and the IgG titer started to increase about a week later in calves aged 6-7 weeks. It is possible that older cattle have a faster IgG response to infection. The maximum titer of IgG was reached between 45 and 76 days after inoculation[96,105] after which it gradually decreased and reached baseline levels around 100 to 140 days after inoculation (i.e. 2-3 months after peak IgG titer)[105].

2.3.4 Carrier animals

Cattle that have recovered from acute *Salmonella* Dublin infection, but probably also animals that have been infected with low doses of bacteria and that have not shown any symptoms, may become carriers of the bacteria[35,96]. Richardson (1973)[92] described 3 types of carriers: Passive, latent and active carriers. Passive carriers have ingested *Salmonella* Dublin bacteria, which merely pass through the gut without invading the intestinal epithelium. These animals usually stop shedding bacteria when they are removed from the contaminated environment or other excreting animals. In latent carriers, the bacteria have left the gut, disseminated to other parts of the animal – usually the lymph nodes, liver, gall bladder and spleen – where the bacteria may persist intracellularly, partly protected from the host defence mechanisms and antibiotic treatment. In such latent carriers the infection may become reactivated with renewed shedding of bacteria months to years after initial infection. Thus, these animals are important for the spread and persistence of *Salmonella* Dublin in infected herds, and for the transmission between herds. Active carriers usually have the bacteria in both the gut and internal organs and continuously excrete bacteria for extended periods of time, sometimes for life. Both active and latent carriers may cause congenital infection of their offsprings probably through transplacental infection. Transplacental infec-

tion in carriers may cause abortion, birth of stillborn calves or birth of weak calves, but it cannot be ruled out that congenitally infected calves may be born as latent or active carriers that may be able to infect their own offspring later in life[47,92].

High numbers of bacteria may be shed from carrier animals both in the faeces, uterine contents and in milk. Between 10^2 to 10^6 bacteria per gram faeces[34,104,106] and 10^2 to 10^5 bacteria per ml milk from one or more quarters of the udder have been reported from natural carriers found under field conditions[108]. Excretion is often intermittent.

Attempts to identify immune mechanisms and serological responses characteristic of carriers have had varying success. Before enzyme-linked immunosorbent assays (ELISAs) became available for detection of a wide range of immunoglobulins directed against different antigens from the salmonella bacterial wall (e.g. LPS, whole cell antigen suspension, flagellar antigens)[17], very little correlation was found between serological responses and long term infection[91]. Through the 1990s several studies have suggested methods for detection of carriers by the means of antibody detecting ELISAs, based on the findings that persistence of the bacteria in carriers frequently leads to continued stimulation of antibody production by the humoral immune system[54]. One study suggested that two serum samples drawn with a 60 day interval for detection of IgG in an ELISA were useful for prediction of the carrier state[108]. However, a later study by the same research group suggested that three serum samples taken over 120 days were required to distinguish carriers from recovered animals by measuring the IgG response[104]. It should be kept in mind that not all animals with persistently high antibody titers against *Salmonella* Dublin can be found culture positive at post mortem examination[51]. These authors suggested that the culture negative high antibody responders may be either false negative in the culture procedure, or the persistence of antibodies may be due to continued stimulation of the specific humoral immune response through memory T-helper cells. Together, the studies suggest that serological identification of carriers by detection of immunoglobulins (in particular IgG) by ELISAs may be a useful and fairly sensitive method, but that it lacks some specificity leading to false positive results. This specificity is difficult to quantify due to the limited sensitivity of culture methods for detection of *Salmonella* Dublin.

Few studies have examined what causes cattle to become carriers. Typhoidal infections may lead to a chronic carrier state in humans, and it has been suggested that the carrier state is a frequent consequence of ingestion of a small inoculum[35]. The same may be the case for

Salmonella Dublin in cattle. Several studies have suggested liver fluke (*Fasciola* hepatica) infestation to be a risk factor for *Salmonella* Dublin infection in cattle in general. Further, it was found through experimental infection with *Salmonella* Dublin in heifers with and without liver flukes that the parasite infestation and concurrent liver tissue damage not only increased the susceptibility of the animals to *Salmonella* Dublin, but also predisposed the animals to become carriers[3,4]. In an observational field study, *Salmonella* Dublin septicemia in five young calves was found to lead to a systemic and mammary gland carrier state as heifers[53]. An experimental study showed that corticosteroid injections 7 weeks after inoculation of *Salmonella* Dublin into the mammary gland lead to recrudescence of bacterial excretion and lymphatic spread of the bacteria to the regional lymph nodes[107]. Together these studies suggest that immunosuppression and concurrent disease in *Salmonella* Dublin infected cattle may increase the risk that animals become carriers. This is the basis for the studies in Chapter 5 of this thesis where it is examined if certain management, environmental and host factors at time of infection influence the risk that animals become carriers as opposed to clearing the infection.

2.4 Transmission pathways

Transmission pathways for *Salmonella* Dublin can be divided in between-herds and within-herds transmission pathways. Between-herd transmission of the pathogen is mainly important for external biosecurity of the individual herds. Within-herd transmission is very important for the infection dynamics of the pathogen leading to certain clinical pictures, health reduction and economical impact in individual herds regardless of whether the infection is endemic in the herd or newly introduced. The transmission pathways within uninfected herds are only important once the bacteria is introduced to the herd. Therefore prevention of introduction of *Salmonella* Dublin plays a major role in these herds. Once *Salmonella* Dublin is introduced to a herd, the speed of dissemination of the pathogen is strongly dependent on management and structure of the barn sections.

2.4.1 Between-herd transmission

The introduction of *Salmonella* Dublin into herds have been examined by several risk factor studies and by studying the possibility for transmission of the bacteria via vectors and infected cattle. In general, the more closed the herd, the lower the risk of introduction of the infection[117]. Trade with *Salmonella* Dublin infected cattle is known to be the major cause of introduction of the bacteria to herds[116], either by direct transfer from an infected herd or by

infection acquired in transit or through dealers[140,141]. The risk of introduction of salmonella bacteria was shown to be four times higher when cattle was brought via markets or dealers than if transported directly between herds[26]. Carriers pose an important risk when moved from their original herd, because the stress of transportation and change of environment, feed etc. may lead to faecal shedding of high numbers of bacteria with no concurrent symptoms. Sharing of pastures by cattle from different herds or contamination of pastures by slurry from infected herds have been documented an important risk factor for introduction and disease outbreaks with *Salmonella* Dublin in cattle herds[75,113]. However, some studies suggested that pasture contamination and sharing of grazing areas were not associated with significantly higher risk of infection in cattle herds[112,116].

Transmission between herds may also occur with vectors or machinery. Vectors are hosts that can carry the bacteria either in faeces or externally on boots, clothing, tools, fur etc. Such vectors may be cats, dogs, rats, birds and wild life. According to Gibson (1965)[33] rats and mice do not play a major role in the spread of *Salmonella* Dublin compared to other types of salmonella bacteria such as *Salmonella* Typhimurium DT104[22,23]. People may also carry and spread the infection between herds. In one study it was possible to isolate salmonella bacteria from contaminated rubber boots 48 hours after casual rinsing[60]. Professionals that are in contact with many herds and many animals during the day pose the highest risk, and veterinarians and inseminators have been suggested as vectors of *Salmonella* Dublin[13,135].

2.4.2 Within-herd transmission

The transmission of *Salmonella* Dublin within herds vary considerably between herds and depends on the structure and separation of different barn sections, the stocking density, the movement of animals through the herd, and management related to hygiene and calving procedures. Carriers are important for the infection dynamics within the herd. Transmission from adult carriers to calves has been described[92] and plays a role mainly around the time of calving when contact between carriers and calves is most intense. In this situation the carriers are subject to stress, which may lead to reactivated infection or increased excretion of bacteria[59,107]and calves are highly susceptible after birth[21,31]. Carriers do not only pose a risk to their own calves in the calving environment. If no measures are taken to avoid cross contamination to the next calving cows and their calves, these may also become infected.

Furthermore, excretion from carriers contaminates the environment both indoors in barns and on pasture.

Single housing is often recommended for young calves and all-in-all-out systems with thorough cleaning and disinfection between batches of calves kept in groups. *Salmonella* Dublin can survive well in faecal matter, and transmission between individually housed calves has been shown to primarily occur via passive transfer between pens on utensils contaminated with infected faeces[44]. Infection is also possible via aerosols[127]. Therefore high pressure cleaning of buildings and pens is a high risk procedure, if live animals are present, or the buildings are not allowed to dry well before new susceptible animals enter the area.

2.5 The Danish National Surveillance program for *Salmonella* Dublin

A national surveillance program for *Salmonella* Dublin in Danish cattle was launched in October 2002 by initiative of both the Danish Veterinary and Food Administration and the Danish Cattle Federation[8]. The short-term purpose of the program was to screen both dairy herds and beef cattle herds for presence of *Salmonella* Dublin infection and to classify the herds according to estimated level of infection in order to provide a control scheme of new infections in Danish cattle herds. The long-term purpose was to reduce the prevalence of *Salmonella* Dublin in Danish cattle and reduce the risk of human infection upon consumption of Danish cattle meat and dairy products.

2.5.1 Classification levels

In the surveillance program all Danish cattle herds are classified into three levels[7]. Dairy herds are classified using bulk tank milk antibody response every quarter of the year. The level is determined from 4 consecutive LPS-ELISA measurements with at least one month between each sample. The average ODC% (the proportion of a background-corrected optical density value of the sample to a known positive control sample) is calculated from these 4 measurements. Non-milk producing herds are classified based on 3 yearly blood samples from animals above 8 months of age, which are collected routinely for evaluation of the herd status of bovine virus diarrhoea (BVD) or voluntarily submitted for analysis.

Level 1 is considered most likely free of *Salmonella* Dublin. Dairy herds are classified as level 1, if the average ODC% of the last 4 bulk tank milk measures is below 25 and no major rise (an increase of 20 ODC% or more) has occurred between the last measurement and the average of the last three samples. For non-milk producing herds all blood samples

must have an ODC% below 50. Levels 2 and 3 are divided into two sub-levels each. Level 2a is "most likely infected" according to antibody responses above the cut-off values described for level 1. Level 2b is not classifiable due to lack of data. Herds in level 3a have diagnosed clinical salmonellosis (usually outbreak herds), and level 3b has had the bacteria detected by culture, but clinical salmonellosis has not been diagnosed. Level 2a is determined solely on antibody measurements from a bulk tank milk ELISA and ELISA response in blood samples. Level 3 is determined solely on microbiological and concurrent clinical findings. The surveillance program has increased the focus on the infection tremendously and caused a high demand for knowledge about how to interpret test results, in particular bulk tank milk ELISA responses in relation to the dynamics of the infection within the herds, and interpretation of individual test results in connection with control strategies in infected herds. This is part of the motivation for the test validation analyses of Chapter 4.

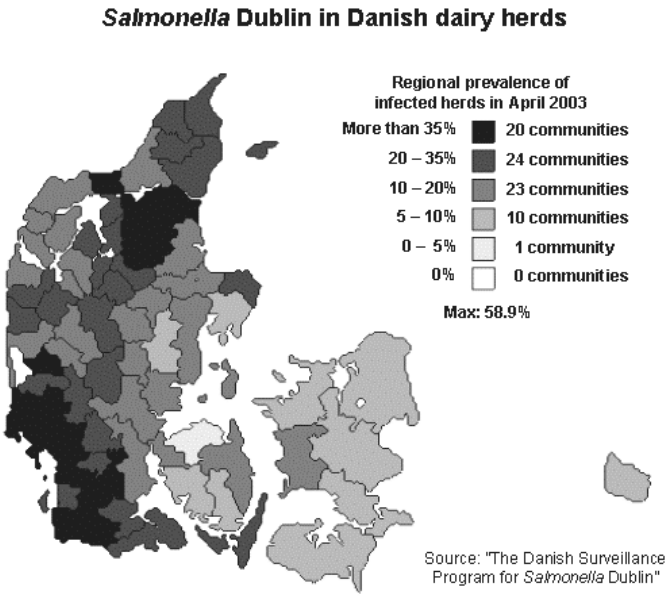


Figure 2.2 Regional prevalence of *Salmonella* Dublin in dairy herds in Denmark according to bulk tank milk ELISA response measured every three months by the Danish National Surveillance Program for *Salmonella* Dublin.

2.5.2 Regional distribution of *Salmonella* Dublin in Denmark

One advantage of the national surveillance program for *Salmonella* Dublin is that it makes it possible to follow the distribution of the infection in more detail. Before the program started the knowledge about prevalence and clinical cases mainly consisted of official registrations based on results from submitted samples of suspect cases, which strongly underestimates the prevalence of *Salmonella* Dublin infected herds, especially those that are endemically infected. Figure 2.2 shows the prevalence of bulk tank milk positive herds according to recordings from the National Surveillance Program for *Salmonella* Dublin in April 2003.

Chapter 3: Materials and methods

The work described in this thesis was part of a large project known as the "Integrated Cattle Health and Milk Quality Project" (also called the Kongeå-project), which was initiated by the Danish Dairy Board in 1997[5] and was based on dairy herds from a region of four postal zip codes in the southern part of Denmark. The map in Figure 3.1. shows the location of the Kongeå-region in Denmark.

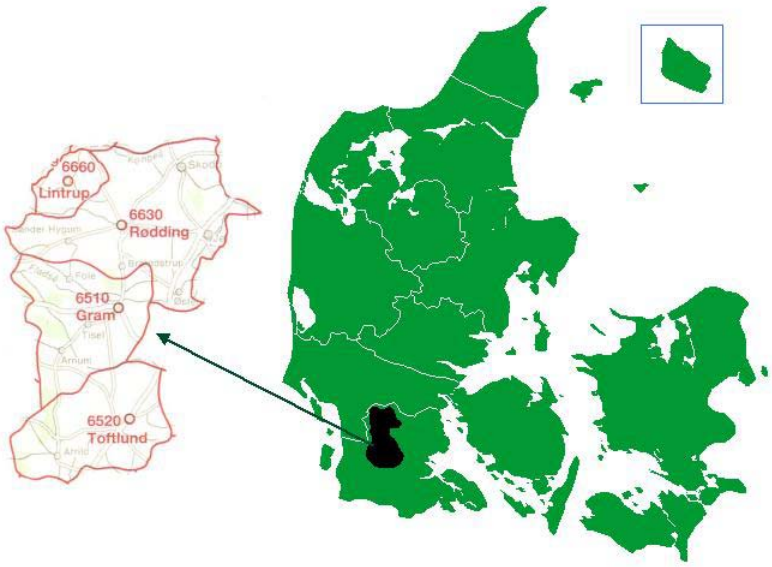


Figure 3.1 All herds participating in the present work came from the Kongeå-region in the southern part of Jutland, Denmark. They participated on a voluntary basis in the "Integrated Cattle Health and Milk Quality Project" (source: the Kongeå-project).

A total of 249 herds were given the opportunity to participate in the Kongeå-project in 1998. The herds were located within 4 postal area codes (6510 Gram, 6520 Toftlund, 6630 Rødding and 6660 Lintrup) in a region of Southern Jutland[5]. A total of 111 herd owners volunteered to participate in the part of the Kongeå-project concerning infectious diseases (paratuberculosis, salmonellosis, Streptococcus uberis mastitis and Escherichia coli O157 infection). The criteria for a herd to participate were that the herds allowed one initial visit by milk-quality advisors (MQAs) to make a set of base-line recordings, and that they gave

permission to use data and results from the herds for both research and advisory purposes. They also needed to be part of the milk-recording scheme to give easy access to routinely recorded production data from the Danish Cattle Database through this program. The herds were consecutively asked permission for every individual sub-project they participated in.

The PhD thesis is based on four major sampling activities during the years 1999-2003. The sampling was performed by MQAs from the Danish Dairy Board (now the Danish Cattle Federation), local veterinarians, three veterinary students, three PhD students, and the laboratory staff at the Danish Cattle Health Laboratory (DCHL) (since 2001 the Veterinary Department, Steins Laboratory) in Ladelund, located just north of the Kongeå-region. The sampling activities provided bulk tank milk samples from a total of 111 dairy herds, of which 94 were sampled monthly throughout the period March 2000 to February 2003 (sampling activity number 1). The remaining 17 herds stopped milk production during the project period. Individual samples and environmental samples were collected from 35 of the dairy herds. The overall study design, the selection of herds, sampling activities, laboratory procedures and the resulting database is described in detail in this chapter, which provides the documentation for the data used in the studies presented in Chapters 4 and 5, as well as the manuscripts.

3.1 Choice of study design

As mentioned in Chapter 1, the overall aim of the thesis is to provide a solid foundation for interpretation of traditional diagnostic tests and studies of within-herd dynamics of *Salmonella* Dublin. Therefore the study design and sample collection scheme were organised so that they would provide data for both the diagnostic test validation studies and risk factor studies in the present thesis, but also for studies of within-herd infection dynamics to be performed after the PhD period.

As illustrated in Figure 3.2 the overall study design used in the present work was a prospective, longitudinal design with repeated measurements. The data structure was multilevel with recordings at herd level, animal level, location/age group level and time level as illustrated in Figure 3.3. This structure was selected to provide the data required to (i) make inference about diagnostic test validity for *Salmonella* Dublin infection in cattle, (ii) determine risk factors for *Salmonella* Dublin infection in individual animals, and (iii) future

estimation of transmission parameters and variables needed for modelling of *Salmonella* Dublin infection dynamics.

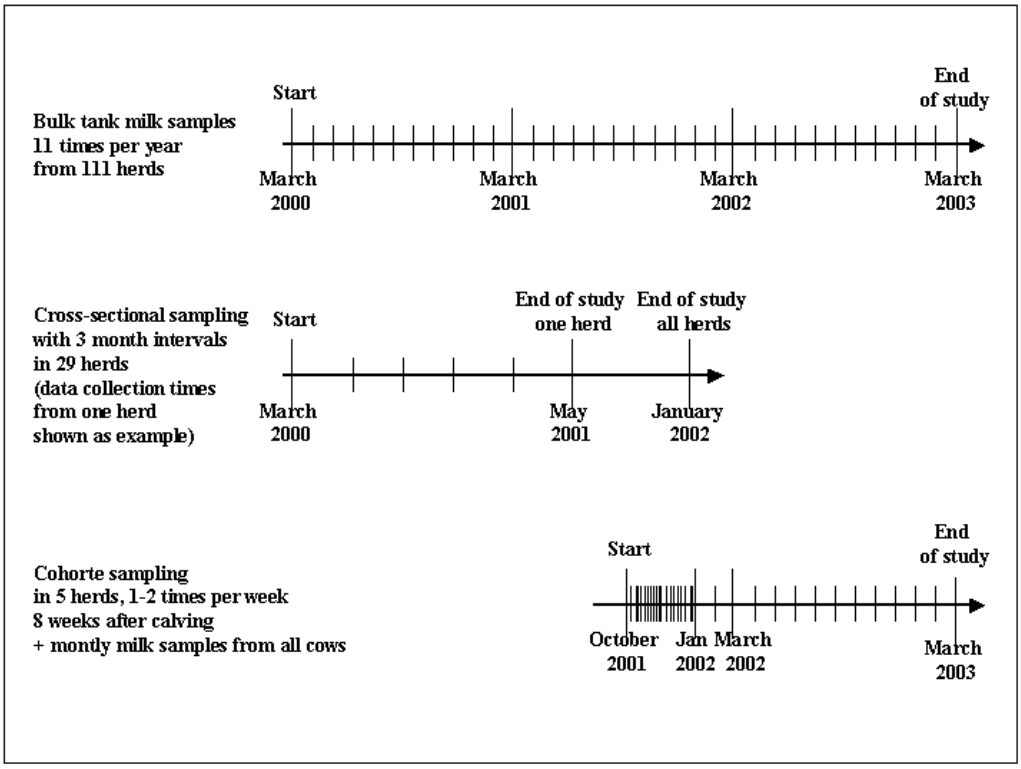


Figure 3.2 Prospective longitudinal design with repeated sampling.

3.2. The CASADY database

CASADY is short for the Cattle Salmonella Dynamics database and consists of datasets containing laboratory results and register data that may be used for a wide range of epidemiological analyses of *Salmonella* Dublin infection in dairy herds either alone or in combination. CASADY contains bacteriological culture results from collected faecal samples and environmental swabs, ELISA results from individual blood and milk samples, ELISA results from bulk tank milk samples, and reproduction and production data from the Danish Cattle Database from the herds participating in the four sampling activities. All sampling activities were planned, initiated and coordinated by the author of this thesis.

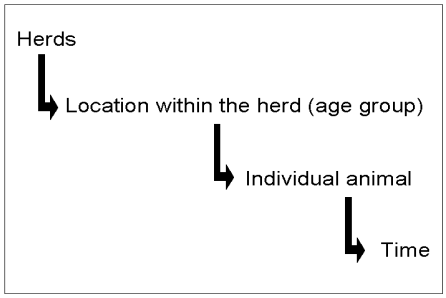


Figure 3.3 Overall data structure for the work presented in the present PhD thesis.

The sampling activities, CASADY construction, data quality control and documentation constituted a major part of the PhD project. CASADY contains explanation and documentation for dataset contents, variable names and laboratory procedures, and is thus ready for use in future studies apart from the work presented in this thesis. In the remainder of this chapter, the background for and structure of the CASADY database is described in detail. Figure 3.4 illustrates the structure of CASADY, and the relations between the sampling activities and herd selections. Figure 3.5 illustrates the contents of resulting datasets of CASADY from the four sampling activities and the collection of register data from the Danish Cattle Database. Appendix A provides more detailed variable explanations from these datasets. Figure 3.14 (page 66) illustrates how the datasets of CASADY were used in combination for the manuscripts and studies presented in Chapters 4 and 5.

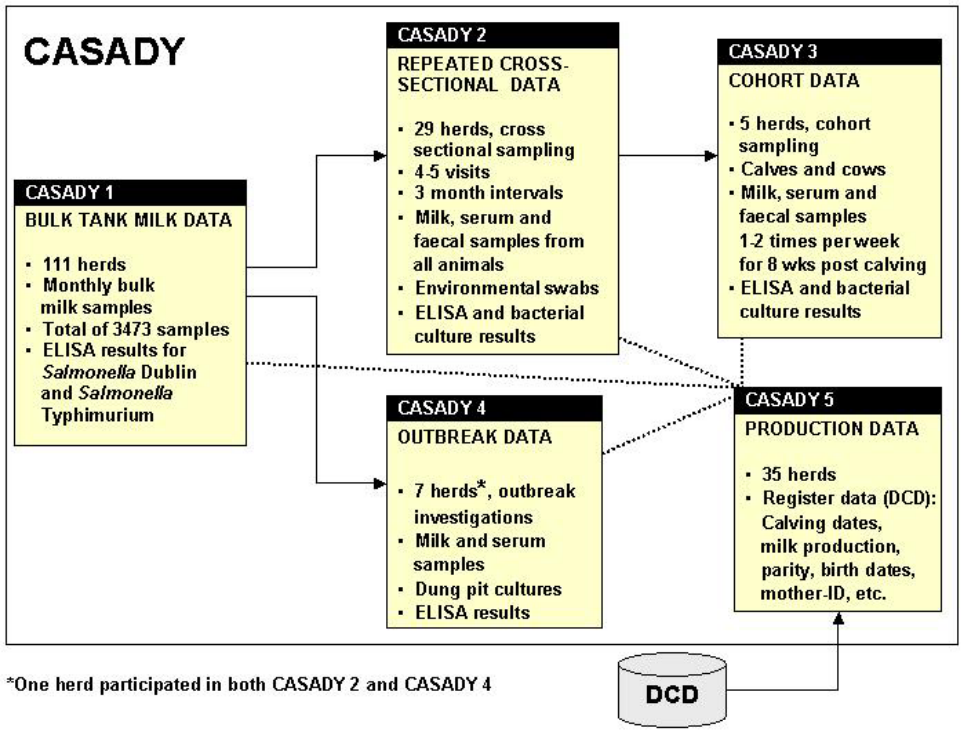


Figure 3.4 An overview of the structure of the CASADY database and the relation between individual datasets. Examples of the contents of the datasets are illustrated in Figure 3.5. Arrows indicate selection of a number of herds from one dataset to another. Dashed lines indicate that register data is available from CASADY5 from the herds and animals. DCD is short for the Danish Cattle Database.

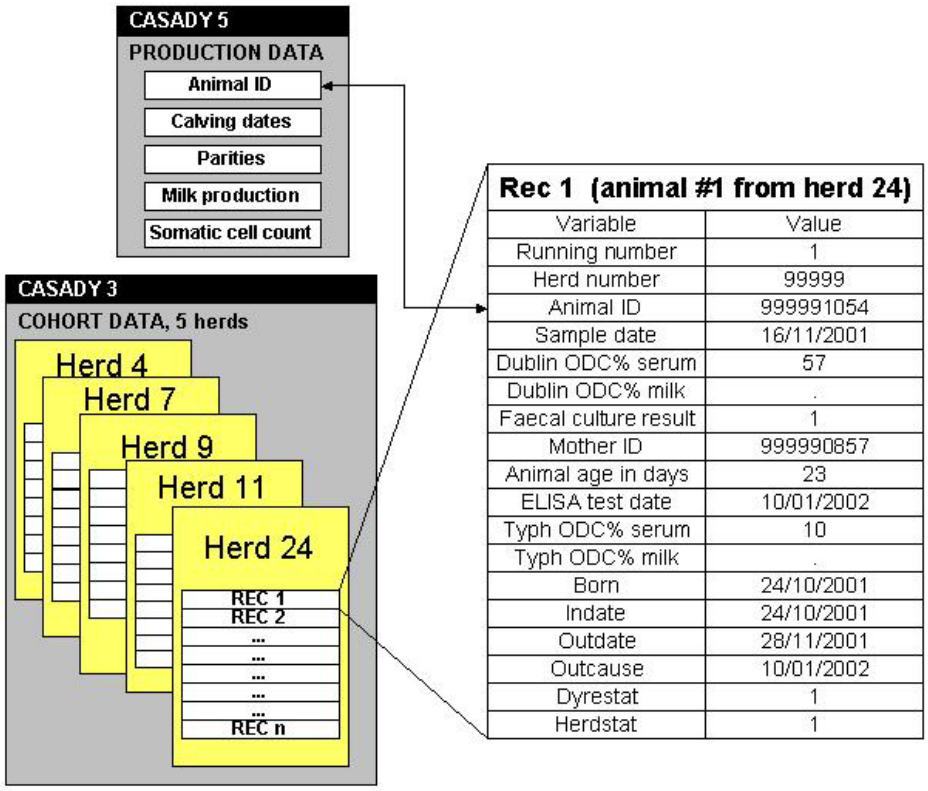


Figure 3.5 Examples of the contents of the datasets CASADY3 and CASADY5. The variables are equivalent in CASADY2 and 4, but the time intervals between sampling and the type of sampling (e.g. cross-sectional vs. cohort) vary. Variable explanation is included in Appendix A.

3.2.1 Herd selection

There were two PhD projects regarding salmonellosis in the Kongeå-project[5]; one microbiology project mainly concerned with development of new and more sensitive diagnostic tests and characteristics of both *Salmonella* Dublin and *Salmonella* Typhimurium bacteria, and the present epidemiology project, which focused on *Salmonella* Dublin. Sampling activities for the two PhD projects were highly coordinated and almost fully overlapping in the first 29 participating herds. Sampling activities were also partly coordinated and overlapping in 6 herds with a paratuberculosis epidemiology project[82].

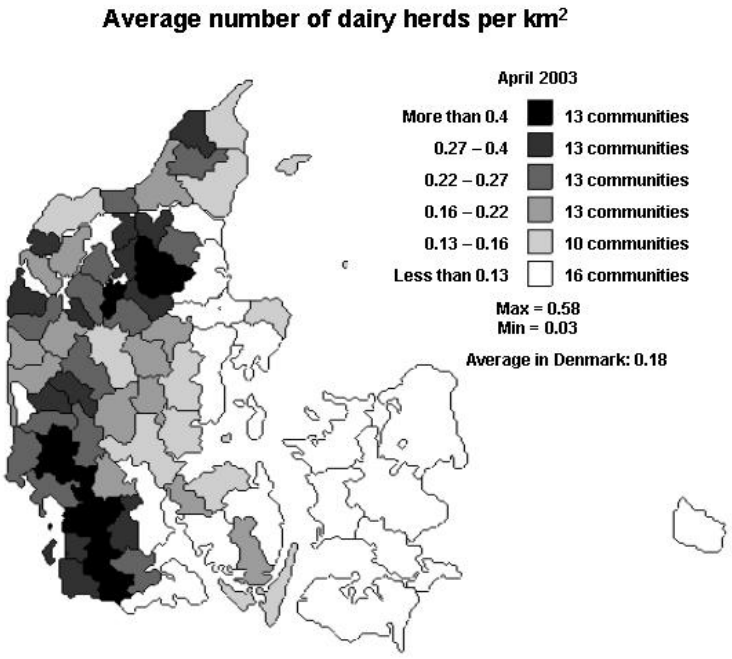


Figure 3.6 An overview of the density of dairy herds in Denmark in April 2003. The Kongeå-region is one of the regions with the highest cattle density (measured as number of herds per km^2) in Denmark.

The Kongeå-region is considered endemically infected with *Salmonella* Dublin. By endemic is meant that the infection is continuously present in the population, but only clinically recognisable in some animals[12]. Occasionally, it causes new outbreaks in susceptible herds. As previously illustrated in Figure 2.2, more than 20% of the dairy herds in the region had positive bulk tank milk antibody responses in April 2003. Similar results were found in a study from 1994 to 1996[131]. The reason for this is probably that the region is one of the parts of Denmark with the highest density of cattle[133], see also Figure 3.6.

It may be relevant to consider to which degree the selected herds represent the full population of interest. In particular, when allowing voluntary participation in epidemiological studies or when selecting herds based on fixed criteria instead of randomly selecting herds from the full population. One such comparison showed that the participating dairy herds were significantly larger (mean herd size: 85 cows) than both non-participating dairy herds from the Kongeå-region (mean herd size: 65 cows) and all diary herds in the country (mean herd size: 67 cows)[83]. Also, this study found that the mean bacterial count of bulk milk

samples in the period April 1999 to March 2000 of the participating herds was significantly smaller (mean: 8,000) than that of the non-participating herds from the region (mean: 10,300) and all Danish dairy herds (mean: 9,000). The mean somatic cell count (cells per ml) of bulk milk samples from the participating herds (mean: 263,000) in the same period was not found to differ from non-participating herds (mean: 262,000) or in all dairy herds in Denmark (mean: 251,000). Figure 3.7 shows the yearly mean somatic cell count of bulk tank milk samples in the Kongeå-project herds and all Danish dairy herds from 1996 to 2002. Figure 3.8 shows the mean bacterial count for the same period for the same two populations.

Among other reasons, the Kongeå-region was selected for the project because the herds in the region were expected to represent future dairy herds in Denmark[5,83]. This may be true with regard to herd size and management types. However, the regional differences in prevalence of salmonella has to be kept in mind. The Kongeå-region is a high-risk region for introduction of *Salmonella* Dublin to the herds compared to less densely populated cattle regions such as Zealand, Funen and Bornholm. The within-herd dynamics are expected to be similar for herds in different regions when other covariates influencing the dynamics of the infection has been controlled for (i.e. herd size, housing type, stocking density, management).

Figure 3.9 shows the regional mean number of cows per herd. The Kongeå-region is one of the most densely cattle populated regions and the dairy herds in this region are among the largest in the country.

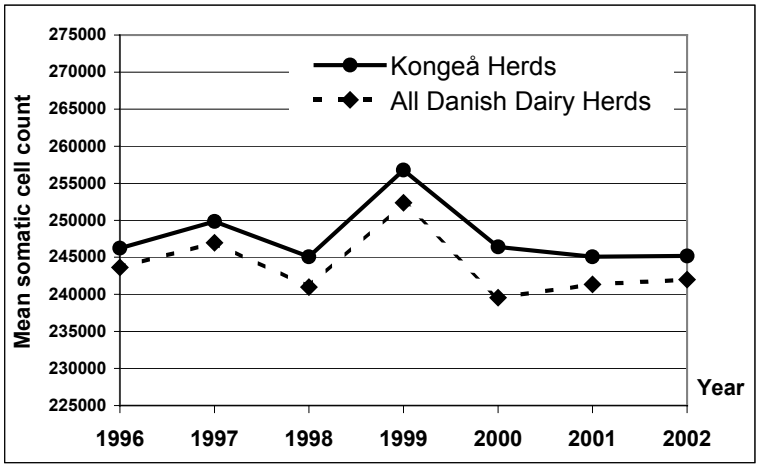


Figure 3.7 Geometrical mean somatic cell count in weekly tested bulk milk samples from Kongeå –project participating herds and all Danish dairy herds (data provided by "MilkInfo" from the Danish Cattle Federation).

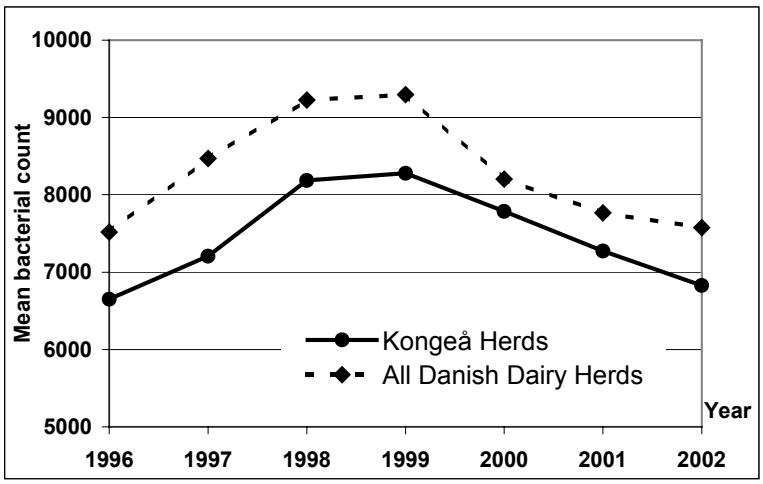


Figure 3.8 Geometrical mean bacterial count in weekly tested bulk milk from Kongeå –project participating herds and all Danish dairy herds (data provided by "MilkInfo" from the Danish Cattle Federation).

.

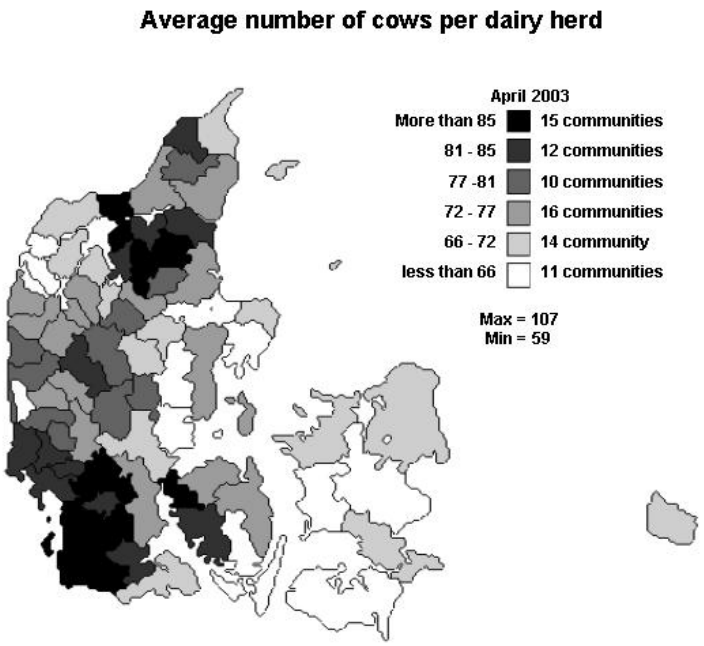


Figure 3.9 Regional mean herd size in Denmark measured as number of lactating cows present in the herds in April 2003 (data provided by "MilkInfo" from the Danish Cattle Federation).

3.2.2 Sampling activities for the CASADY database

The laboratory results used in this thesis all came from four major sampling activities (Act#1 to Act#4) and one register data collection activity (Act#5) with minimal overlap. Figure 3.10 provides an overview of the activities and resulting datasets in each sampling and data collection activity. Detailed description of the datasets and variables can be found in Appendix A. Throughout the sampling activities the samples were handled as follows:

- Milk samples were spun in a centrifuge at 3200 rounds per minute (RPM) for 4 minutes. The fat-fraction was removed, and the remaining sample was kept frozen at -18°C until analysis.
- Blood samples were kept refrigerated at 4ºC for 1-2 days, and then spun at 3200 rpm for 4 minutes. The serum fraction was kept frozen at –18ºC until analysis.

All milk and serum samples were analysed for immunoglobulins against *Salmonella* Dublin and *Salmonella* Typhimurium LPS by the indirect ELISAs described in section 3.3.2.

- Faecal and environmental samples were cultured for salmonella bacteria using the method described in section 3.3.1. From March 2000 to October 2001 the faecal samples were pooled using 5 g of faecal matter from each of 5 individual samples. If a pool was found positive, the individual samples were cultured using 25 g per sample. This method will be referred to as the pool-first culture method in the rest of this thesis. From October 2001 all samples were cultured individually using 25 g (or all material available).

The ELISA results, bacteriological culture results and animal characteristics (e.g. date of birth, date and reason of entrance to and departure from the herd, breed, mother's identification number) from the Danish Cattle Database were entered in one dataset with one sample event per animal and sample date. One sample event consisted of one to four ELISA results (serum and/or milk for *Salmonella* Dublin and/or Typhimurium) and one bacteriological culture result.

Sampling activity 1 – "Bulk tank milk ELISA response over time" (Act#1)

The first sampling activity consisted of collection of bulk tank milk samples from the 111 dairy herds that participated in the Kongeå-project. The samples were collected by the drivers of the dairy milk trucks approximately every fifth week (11 times per year) by the use of specialized equipment to collect the samples in a standardised manner. A total of 3473 samples were collected during the period March 2000 to February 2003. The herds were sampled between 5 and 41 times with a mean of 31.2 times per herd. Of the 111 herds, 94 were sampled throughout the sampling activity period.

Sampling activity 2 – "Repeated cross-sectional study" (Act#2)

Out of the 111 herds, 30 were asked to participate in sampling activity number 2, and 29 agreed to participate. These herds were selected based on bulk tank milk ELISA responses against *Salmonella* Dublin and *Salmonella* Typhimurium LPS in March-May 2000 and willingness to participate in fairly extensive sampling activities every three months for more than a year. The herds were selected to cover the full range of bulk tank milk ELISA responses, thus attempting add sufficient variation to the herds with regard to within-herd dynamics (Table 3.1). A relatively high number of herds with medium to high ELISA responses against *Salmonella* Dublin was chosen, because it was considered important to

include enough herds for within-herd infection dynamics studies of *Salmonella* Dublin. The bacteria had to be present in the herd for this purpose. Appendix B provides an overview of each of the 29 herds with regard to herd size, breed, barn systems etc. at time of selection.

Table 3.1 Selection criteria for herds participating in sampling activity number 2 (quarterly visits 4-5 times over 1-1½ years).

Level and type of bacteria	**ELISA response against *Salmonella* Dublin LPS**	**Number of herds**
High Dublin	Continuously > 50 ODC%	11
Medium to high Dublin	Varying between 30 to 80 ODC%	5
Low to medium Dublin	15 to 65 ODC%	7
Low Dublin	< 15 ODC%	4
High Typhimurium / Outbreak of Typhimurium	> 100 ODC% *	2

* For these herds selection criteria were based on bulk tank milk ELISA response against *Salmonella* Typhimurium

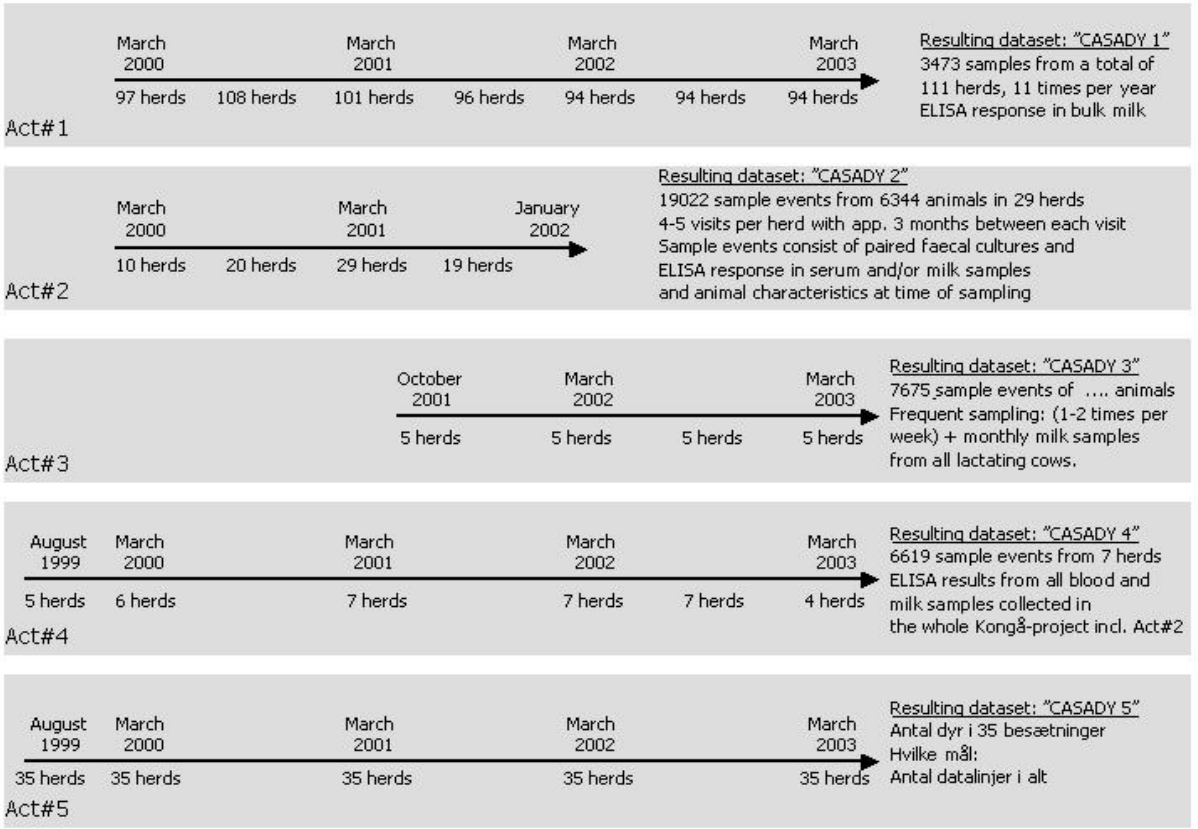


Figure 3.10 An overview of sampling and data collecting activities for CASADY. For more detailed descriptions of the activities, the laboratory procedures, data editing and the resulting datasets see the relevant sections of this chapter.

The 29 herds were visited approximately every 3 months. During the Foot and Mouth Disease outbreak in Great Britain and The Netherlands in Spring 2001 this schedule was not completely kept. All in all, 24 herds were visited 5 times and 5 herds were visited 4 times. The sampling activity provides repeated cross-sectional investigation of all the participating herds. At each visit the following samples were collected: Faecal samples collected rectally from all animals on the premises, 20 environmental swabs (window sills, walls, feeding tables, tank room floor, ventilation holes, etc.) including at least one sample from the dung pits, milk samples from all lactating cows collected during the morning milking routine, blood samples from all non-lactating animals including all calves, young stock, heifers and dry cows. In 3 *Salmonella* Dublin infected herds, blood samples were also collected from lactating cows during one of the 5 visits. At the first visit to each herd, either Birgitte Langvad (PhD student in the salmonella microbiology project under the Kongeå-project) or the author participated in sampling, pointed out spots for environmental swabs and interviewed the herd owner to obtain the history of the herd with regard to salmonellosis. All samples were collected by MQAs and the local practicing veterinarians and brought to DHCL/Steins Laboratory for registration and processing.

The following samples were collected through sampling activity #2 from March 2000 to January 2002:

- 9,775 milk samples (153 to 591 milk samples per herd)
- 9,961 serum samples (161-692 serum samples per herd)
- 19,393 individual faecal samples (276-1363 samples per herd)
- 2,753 environmental swabs

In Table 3.2 the distribution of 137 positive faecal sample results in the 29 herds are shown. In Figure 3.11 the age distribution of these faecal culture positive animals is shown.

Table 3.2 Number and serotype of salmonella (*S.*) bacteria isolated in sampling activity number 2.

Herd no.*	Salmonella positive faecal cultures		Salmonella positive environmental swab		
	n	**Serotype**	**n**	**Serotype**	**Locations**
1	7 1	*S.* Dublin NTS**	2 1 3	*S.* Dublin NTS *S.* Derby	Young stock and dung pit Young stock Young stock
2	8	*S.* Dublin	5	*S.* Dublin	Cow barn and tank room and dung pit
4	5	*S.* Dublin	8	*S.* Dublin	Young stock, calves and dung pit
5	6	*S.* Dublin	4	*S.* Dublin	Young stock, cow barn and dung pit
6	1	*S.* Dublin	0		
7	19 1	*S.* Dublin NTS	9	*S.* Dublin	Cow barn and tank room, calf barn and dung pit
8	1	*S.* Dublin	1	*S.* Dublin	Young stock
9	10	*S.* Dublin	5	*S.* Dublin	Young stock, heifers and calf huts
11	11	*S.* Dublin	0		
13	6 1	*S.* Dublin NTS	1	*S.* Dublin	Young stock
14	14	*S.* Typhimurium (DT170)	12	*S.* Typhimurium (DT170)	Calves, young stock, cow barn, dung pit
15	8	*S.* Dublin	1	*S.* Dublin	Calf single pens
16	8	*S.* Dublin	1	*S.* Dublin	Calf single pens
18	0		1	*S.* Agona	Dung pit in young stock barn
19	9	*S.* Typhimurium (DTU312, DT193, DT120)	10	*S.* Typhimurium (DTU312, DT193, DTU302, DT120)	Dung pit, young stock barn, calf pen area
20	5	*S.* Dublin	9	*S.* Dublin	Calves, young stock, dung pit
24	12	*S.* Dublin	7	*S.* Dublin	Calves, young stock, heifers, dung pit
25	4	*S.* Typhimurium (DT 17)	1	*S.* Typhimurium (DT 17)	Dung pit

* The herd number refers to the herds described in Appendix B

**NTS = non-typable salmonella

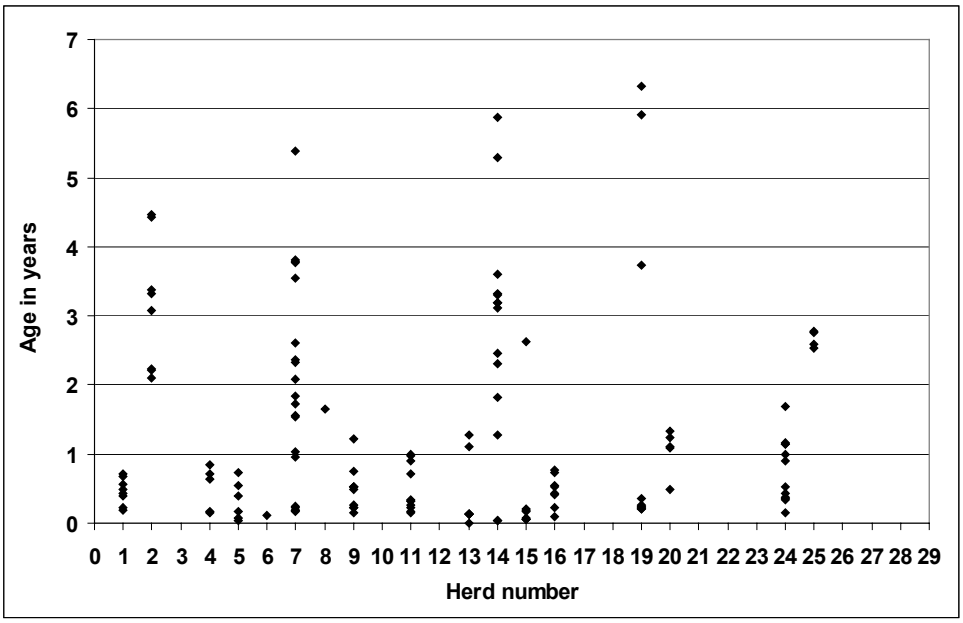


Figure 3.11 Age distribution of *Salmonella* positive faecal samples found in 29 herds in sampling activity number 2.

Extensive data control and editing was performed to reduce the number of erroneous registrations. A total of 714 sample events of 19,736 (3.6%) were disregarded due to unrecoverable human and/or technical errors, such as (i) errors in registration of animal ID-numbers, (ii) large variation in ELISA-duplicate wells not improving with retesting, (iii) lack of simultaneous bacteriological culture and ELISA results. Thus 19,022 sample events from 29 herds are contained in the final dataset from sampling activity number 2. All in all, 6344 animals were sampled. Of these, 1317 were sampled once, 1050 twice, 1435 three times, 1410 four times and 1132 were sampled five times.

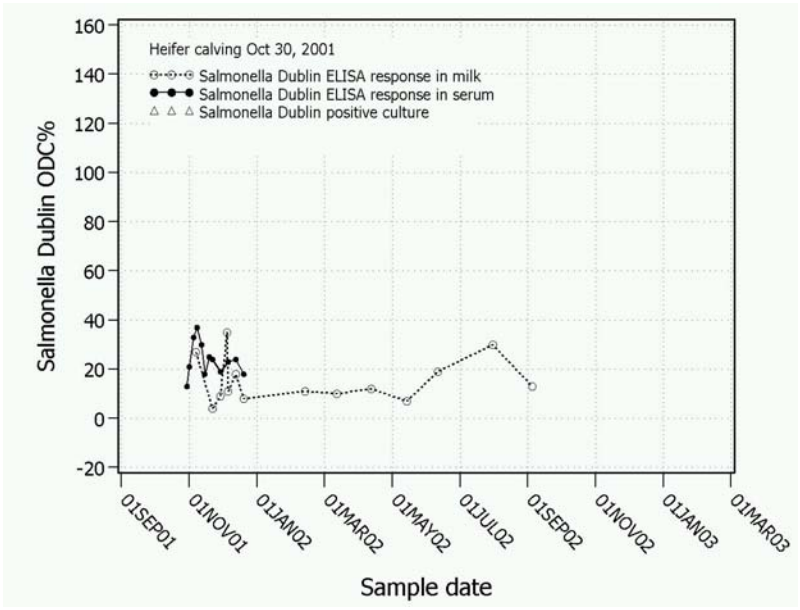
Sampling activity 3 – "Calving project" (Act#3)

In infection dynamics models it is often necessary to determine the infection status of each animal contributing to the model parameters frequently enough that only one new infectious animal generation could have emerged between to sample events. SIR models are useful and illustrative models for rapidly spreading infections[37]. However, data collection becomes very work intensive and thus expensive. Sometimes this problem is solved using set-ups with highly controlled experimental conditions. These types of studies, however, may not

lead to conclusions transferable to real-life situations. Thus, in this sampling activity it was attempted to measure the actual transmission patterns in 5 of the 29 herds participating in sampling activity number 2. Because of the relatively short incubation time for *Salmonella* Dublin, the animals were sampled with faecal samples, serum and milk samples once to twice per week. It was not economically and practically feasible to sample all animals in the herds this often, so a cohort of animals was selected for the study. The cohort consisted of all cows and heifers calving in the period October 10th to December 5th 2001 and their calves. All neighbour calves to the newborn calves were sampled on the same days as the calves, and monthly milk samples were collected through the milk-recording scheme from all cows from September 2001 to March 2003. It was expected that this sampling scheme would make it possible to study the spread of infection around time of calving and in the barn section for young calves. Also new knowledge about the dynamics of infection and antibody responses in the cows in endemically infected herds could be obtained from these data. The herds were selected because they were known to have had *Salmonella* Dublin related problems (e.g. periodically increased calf mortality, diarrhoea, pneumonia, abortions) and wide-spread infection in the herds for years, and because *Salmonella* Dublin bacteria were found on several occasions during sampling activity number 2. The following herds agreed to participate: Herd number 4, 7, 9, 11 and 24 (herd numbers refer to Appendix B).

Two teams were collecting samples during this sampling activity. Team A consisted of two veterinary students and the author. Team B consisted of MQAs and the local practicing veterinarians. Team A were on 24 hour call from October 10th to December 5th 2001 in order to sample the cows and calves immediately after calving. The following samples were collected at time of calving: blood sample from the newborn calf before colostrum uptake, blood and colostrum samples from the mother for ELISA analysis. Faecal sample from the mother, samples of colostrum and uterine contents were collected for bacteriological culture of salmonella bacteria. Team B collected blood, milk and faecal samples from the cows and calves in the cohort twice a week in the period October 10th to January 2002. All animals in the cohort were sampled during a period of at least 8 weeks after calving/birth. Registrations were made of clinical signs and exact location in the herd on day of sampling. It was also registered if the calves received anti-*Salmonella* Dublin serum treatment. In the two herds, where this was common practice, only some of the calves received antiserum treatment. From September 2001 to March 2003 monthly milk samples from all lactating cows were collected through the milk-recording scheme.

A total of 3130 paired sample events (blood and faeces or blood, milk and faeces) were collected from the cohort and calf barn sections in the 5 participating herds in the period October 10th 2001 to January 31st 2002. In addition, 5955 milk samples were collected through the milk-recording scheme from September 2002 to March 2003. These were not paired with faecal samples. All faecal samples were cultured both individually for salmonella bacteria using 25 g per sample, and in pools of 5 g from each of 5 samples. If the sample consisted of less than 25 g faecal matter, the entire sample was used for the individual culture. The pooled and individual faecal culture results were used for sensitivity comparison in section 4.2 of this thesis. Figure 3.12a to 3.12c show examples of test results from animals from the cohort study. The data were used for the studies described in Chapter 4 and 5 in combination with laboratory results from sampling activity number 1, 2 and 4 as illustrated in Figure 3.14.



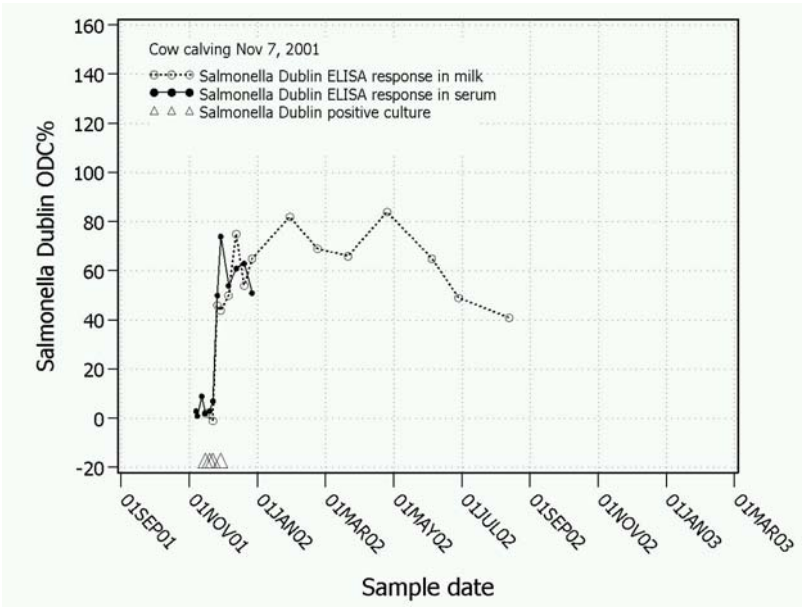
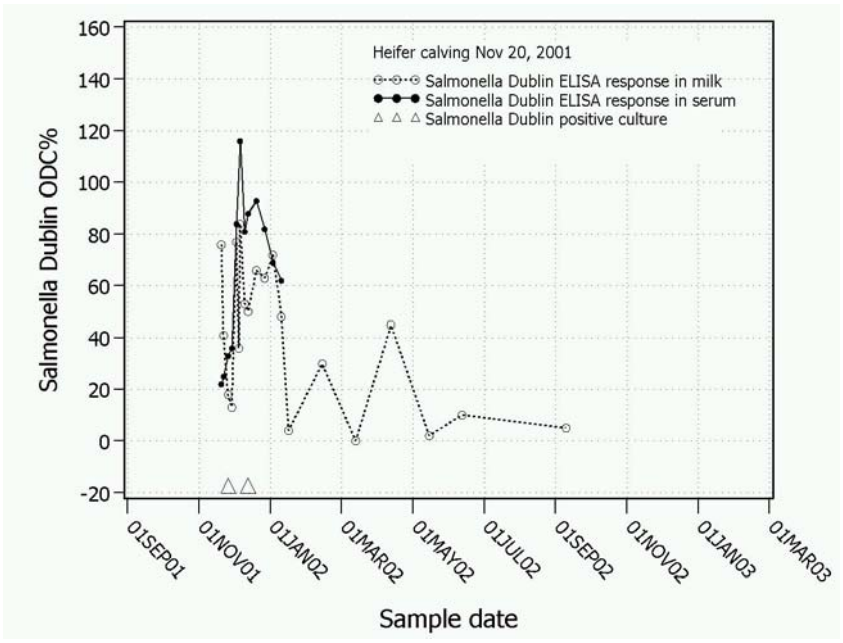


Figure 3.12a (Full figure text on page 57).



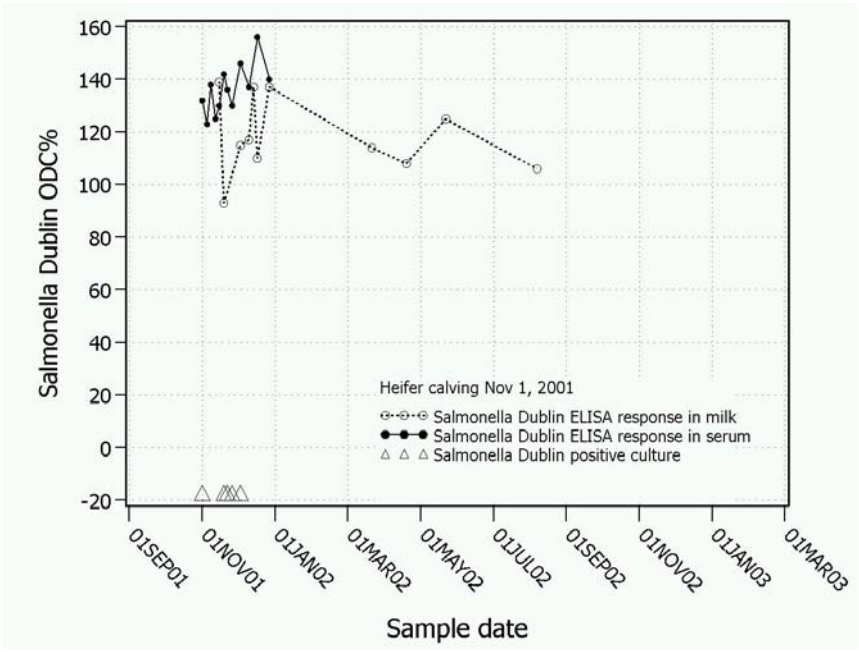


Figure 3.12b (Full figure text on page 57).

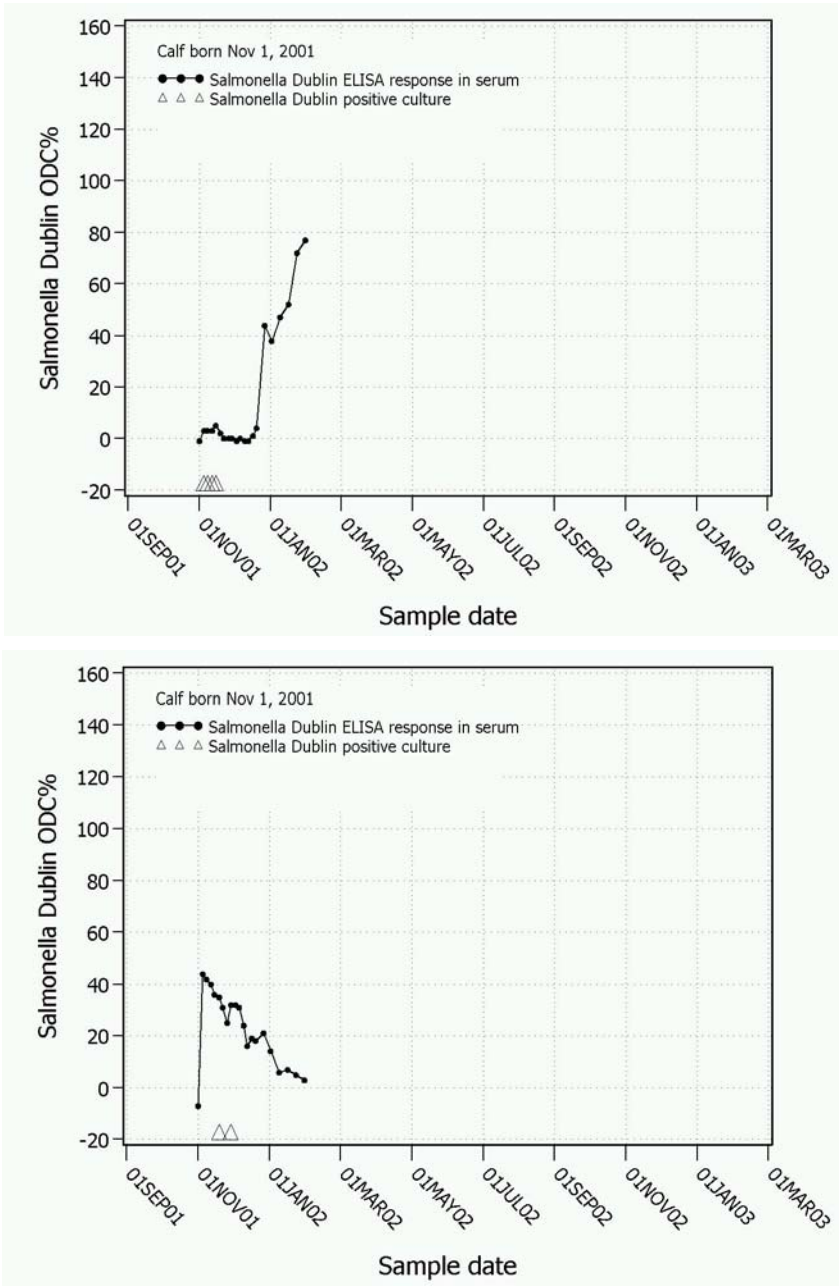
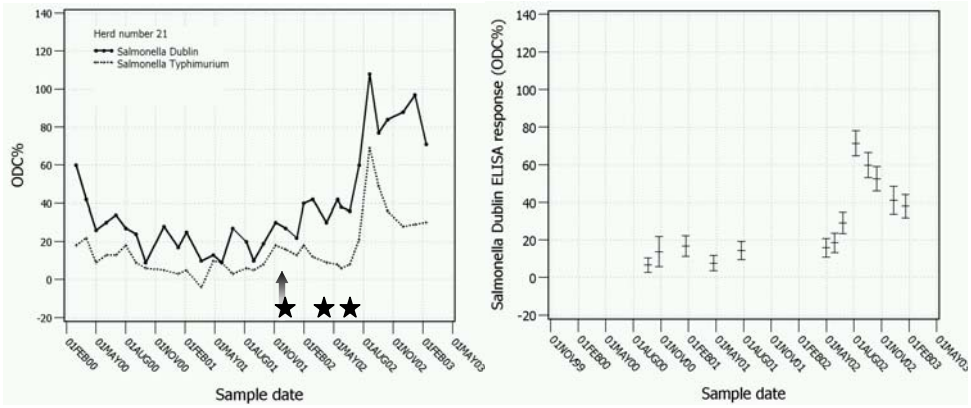


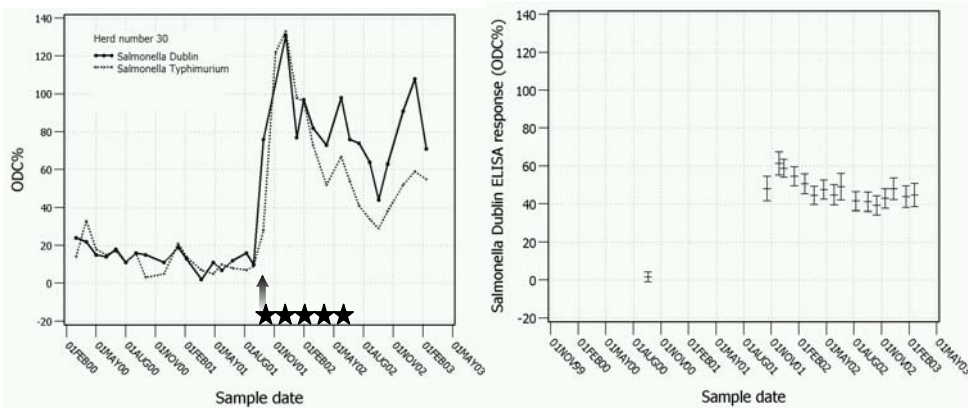
Figure 3.12c

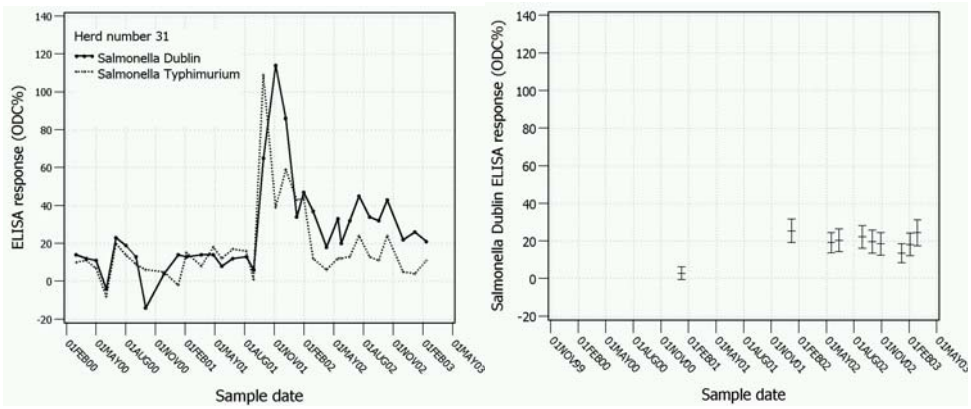
Figure 3.12a-c Examples of bacteriological culture results and ELISA response in serum and milk samples collected from a cohort of animals from time of calving or birth onwards in sampling activity number 3. The first sample from the calves consisted of precolostral blood samples.

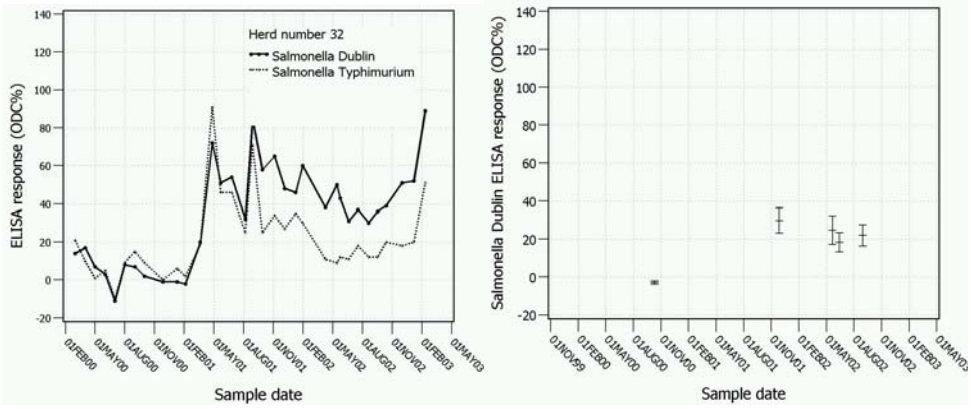
Sampling activity 4 – "Outbreak Investigations" (Act#4)

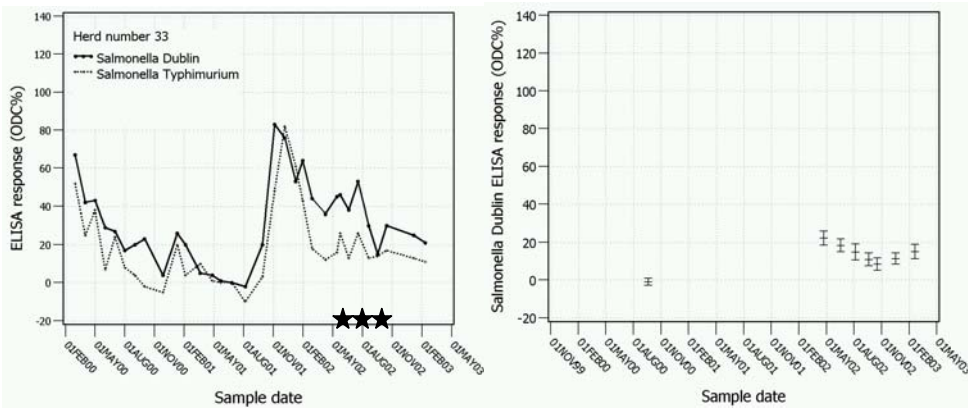
In September 2001 one of the 111 herds participating in sampling activity number 1 experienced a serious outbreak of *Salmonella* Dublin among the cows in a fully susceptible population. Two months later herd number 21 experienced an outbreak of *Salmonella* Dublin among the young calves. This herd was initially selected for participation in sampling activity number 2 based on low bulk tank milk responses against both *Salmonella* Dublin and *Salmonella* Typhimurium, and no salmonella bacteria were cultured during sampling activity number 2. The bulk tank milk antibody responses against *Salmonella* Dublin and *Salmonella* Typhimurium for the two herds are shown in Figure 3.13 (herd 21 and 30). Around the same time, the technical group of the National Surveillance Program that was introduced in section 2.5, was working on determining cut-off values and sampling schemes based on bulk tank milk antibody responses for dairy herds, and more knowledge about bulk tank milk response in relation to new outbreaks of *Salmonella* Dublin was needed. Also, knowledge about how to interpret sudden increases in the bulk tank milk antibody response against *Salmonella* Dublin was required. Therefore, the group sponsored sampling and laboratory analyses of 7 herds from the Kongeå-region. These herds had either been through a known and diagnosed *Salmonella* Dublin outbreak, or had bulk tank milk ELISA responses similar to these outbreak herds. Hence, these herds were likely to be considered as outbreak herds in a surveillance program. The bulk tank milk ELISA responses for the other five herds that were selected for sampling activity number 4 are also shown in Figure 3.13. The selection criteria were that the bulk tank milk response against *Salmonella* Dublin had to be low (below 40 ODC%) for more than 6 consecutive months, followed by an increase of at least 40 ODC% over less than 3 months. Seven herds out of the 111 herds from sampling activity number 1 were selected based on these criteria for the outbreak investigations.

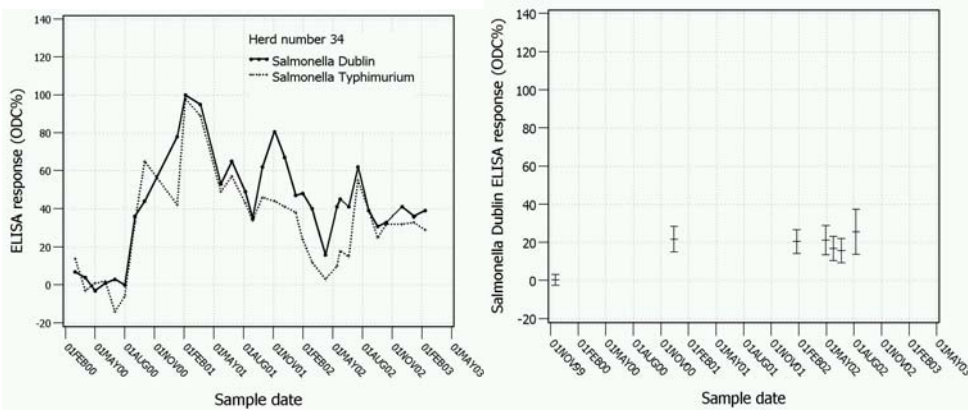

Herd number 21
Salmonella Dublin
Salmonella Typhimurium
ODC%
Sample date
Salmonella Dublin ELISA response (ODC%)
Sample date


Herd number 30
Salmonella Dublin
Salmonella Typhimurium
ODC%
Sample date
Salmonella Dublin ELISA response (ODC%)
Sample date


Herd number 31
Salmonella Dublin
Salmonella Typhimurium
ELISA response (ODC%)
Sample date
Salmonella Dublin ELISA response (ODC%)
Sample date


Herd number 32
Salmonella Dublin
Salmonella Typhimurium
ELISA response (ODC%)
Sample date
Salmonella Dublin ELISA response (ODC%)
Sample date


Herd number 33
Salmonella Dublin
Salmonella Typhimurium
ELISA response (ODC%)
Sample date
Salmonella Dublin ELISA response (ODC%)
Sample date


Herd number 34
Salmonella Dublin
Salmonella Typhimurium
ELISA response (ODC%)
Sample date
Salmonella Dublin ELISA response (ODC%)
Sample date

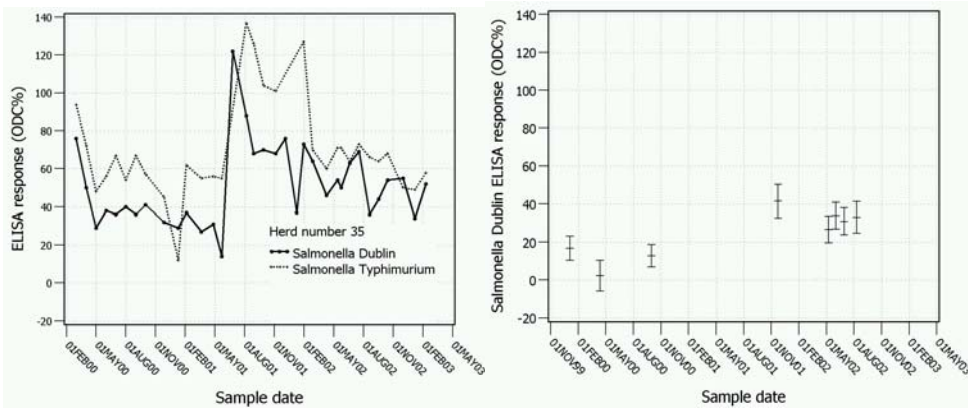


Figure 3.13 In the left column: Bulk tank milk ELISA response against *Salmonella* Dublin (solid lines) and *Salmonella* Typhimurium (dashed lines) in seven herds selected for participation in sampling activity number 4 - "Outbreak investigation" (the herd numbers refer to Appendix B). In the right column: Mean *Salmonella* Dublin ELISA response in individual milk samples with 95% confidence limits. These graphs also show when samples were collected in relation to bulk tank milk rises suggestive of clinical outbreaks. The arrows indicate known time of onset of a clinical outbreak of *Salmonella* Dublin. Stars indicate *Salmonella* Dublin culture positive individual faecal samples or dung pit samples.

The seven herds were visited 3 times in the period April to September 2002, where serum samples were collected from all non-lactating animals above 3 months of age for analysis of antibodies against *Salmonella* Dublin and *Salmonella* Typhimurium. At the same visits dung pit samples were collected for culture for salmonella bacteria at DVI. Monthly milk samples were collected through the milk-recording scheme from April 2002 to September 2002 (for 4 herds until March 2003). All blood and milk samples and laboratory results that had been collected in these herds through this and other PhD projects in the Kongeå-project, were also collected in an attempt to "reconstruct" the infection status of these 7 herds before, during and after the outbreaks (or bulk tank milk rises suggestive of outbreaks). A total of 8512 sample events were collected from individual animals from August 1999 to March 2003. Due to the retrospective gathering of samples and data, the available data vary considerably between herds in relation to the respective disease outbreaks and rises in bulk tank milk responses. Dates for sampling of individual milk samples and mean *Salmonella* Dublin ELISA response with 95% confidence limits are shown in Figure 3.13, to the right of the bulk tank milk measures for the seven herds participating in the outbreak investigations. Figure 3.13 also indicates apparently strong cross reactions of antibodies from the

bulk tank milk in the two ELISAs. Cross reactions in the ELISAs will be discussed in more detail in Chapter 4.

Register data

Animal characteristics, production and reproduction data were collected from the Danish Cattle Database. The data collected for use in CASADY5 contain information about birth date, breed, mother's ID-number, date of entrance to the herd (if different from birth date), date of departure from the herd, reason for departure, calving dates, parity, milk yield, fat and protein contents of the produced milk (used to calculate energy corrected milk yield (ECM)) and somatic cell count.

3.3 Laboratory procedures

3.3.1 Bacteriological culture method

Faecal samples were examined at DCHL/Steins Laboratory for the presence of salmonella bacteria by the following method:
25 g of faecal material (pool of 5 g from each of 5 animals) was mixed in 225 mL peptone buffer and left for pre-enrichment at 37ºC for 18-24 hours. Inoculation of 0.1 ml test material onto Modified Semi-solid Rappaport Vassiliadis Medium Base (MSRV-agar) plates and 1 ml test material into 9 ml of selenite-cystine was followed by incubation for 18-24 hours at 41.5ºC. Material from the selenite-cystine tubes was inoculated on modified Brilliant-green Phenol-red Lactose Sucrose agar (BPLS-agar) plates and incubated at 37ºC for 18-24 hours. Positive test results from MSRV were inoculated onto BPLS-agar plates and confirmed using Triple Sugar Iron agar-tests and Lysine-Iron-agar tests. Serotyping and confirmation of positive isolates were conducted at the Danish Veterinary Institute (DVI).

Environmental swabs were collected by the use of tampons dipped in peptone buffered fluid. The swabs were cultured similarly to the faecal samples except the pre-enrichment occurred by keeping the containers with 1 litre of peptone buffer used for collection of the swabs at 37ºC for 18-24 hours.

3.3.2 ELISA procedures

The serum *Salmonella* Dublin ELISA used in this study was performed at DCHL/Steins Laboratory slightly modified from a previously described ELISA method[50]. An O-antigen based *Salmonella* Dublin LPS preparation produced at DVI was diluted 1:80,000 with 0.1 M sodium carbonate buffer pH 9.6 and used to coat microtitration plates (Polysorb Cat# 475094, Nunc, Denmark) at 4ºC for 2 days (100 µl/well). Plates were blocked using 200 µl/well phosphate buffered saline (PBS) with 1% polyvinylpyrrolidone (PVP) for 30 minutes at room temperature and then washed 3 times on a Skan Washer 300 (Skatron Instruments, Norway) using PBS solution (pH 6.8) with 0.05% $Tween_{20}$. Sera were diluted 1:200 in PBS containing 1% polyvinylpyrrolidone-40T and added to the microtitration plate wells in duplicates using the robot Tecan Genesis RSP 200 (Tecan Nordic, Denmark). Known positive and negative reference sera were added in quadruplicates. The plates were incubated overnight (16-20 hours) at 4ºC, and washed 3 times. For detection of immunoglobulins, affinity purified horseradish peroxidase labelled goat anti-bovine IgG (H+L)

conjugate (Cat# 14-12-06, Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) was diluted 1:6000 in PBS containing 1% PVP, added to all wells in the plate (100 µl/well), incubated for 1 hour at 37ºC and then washed 3 times. 100 µl/well of substrate and indicator solution (100 ml 0.1 M citrate buffer pH 5, 0.3 mg/ml 1.2-orthophenyldiamine dihydrochloride (Kem-En-Tec, Copenhagen, Denmark), 0.5 ml $Tween_{20}$ and 80 µl 30% hydrogen peroxide) was added to the wells and incubated in the dark at room temperature for 10-20 minutes. The reaction was stopped by adding 100 µl/well 0.5 M H_2SO_4 to all wells when the optical density of the positive reference wells was visually evaluated to be approximately 2.000. The optical density (OD) was read at 492 nm and 620 nm as reference using an ELISA plate reader (Labsystems Multiscan MS, Bie & Berntsen, Rødovre, Denmark). An automated plate and duplicate wells validity control was established and used routinely by the author from January 2001. Plates were considered valid if the 4 negative reference wells had an average OD of less than 0.300, and the 4 positive reference wells had an average OD of 1.200-2.500. The size of the variation between the reference wells was also part of the plate validity criteria. The validity of the single samples was evaluated based on the variation between the optical density read from the duplicate wells in relation to the size of the resulting ODC% by the formula, and was rerun if the difference between the duplicate wells were larger than 0.17+0.0016×ODC%. In this way, more variation was allowed in samples containing high concentrations of antibodies than in samples with low concentrations of antibodies. The ODC%-value, which is a background corrected ratio of the test sample OD to a positive reference sample, was calculated for each sample as follows:

$$ODC\% = \frac{\left(\overline{OD}_{sample} - \overline{OD}_{neg\ ref}\right)}{\left(\overline{OD}_{pos\ ref} - \overline{OD}_{neg\ ref}\right)} * 100\%$$

where $\overline{OD}_{sample}$ is the mean value of two test wells, $\overline{OD}_{neg\ ref}$ and $\overline{OD}_{pos\ ref}$ are the mean values of four reference wells in the ELISA plates.

The milk *Salmonella* Dublin ELISA was performed in a similar way, except that milk was added to the wells undiluted and the plates were washed 6 times between steps instead of 3 times. The accept criteria were set slighter higher, because the milk ELISA has more background reaction. Single samples were rerun if the difference between the duplicate well were larger than 0.2+0.002×ODC%.

The *Salmonella* Typhimurium ELISAs were performed similarly to the *Salmonella* Dublin ELISAs except the plates were washed by hand instead of using the Skan Washer. The Typhimurium test was not used routinely at Steins Laboratory, and had not yet been properly validated.

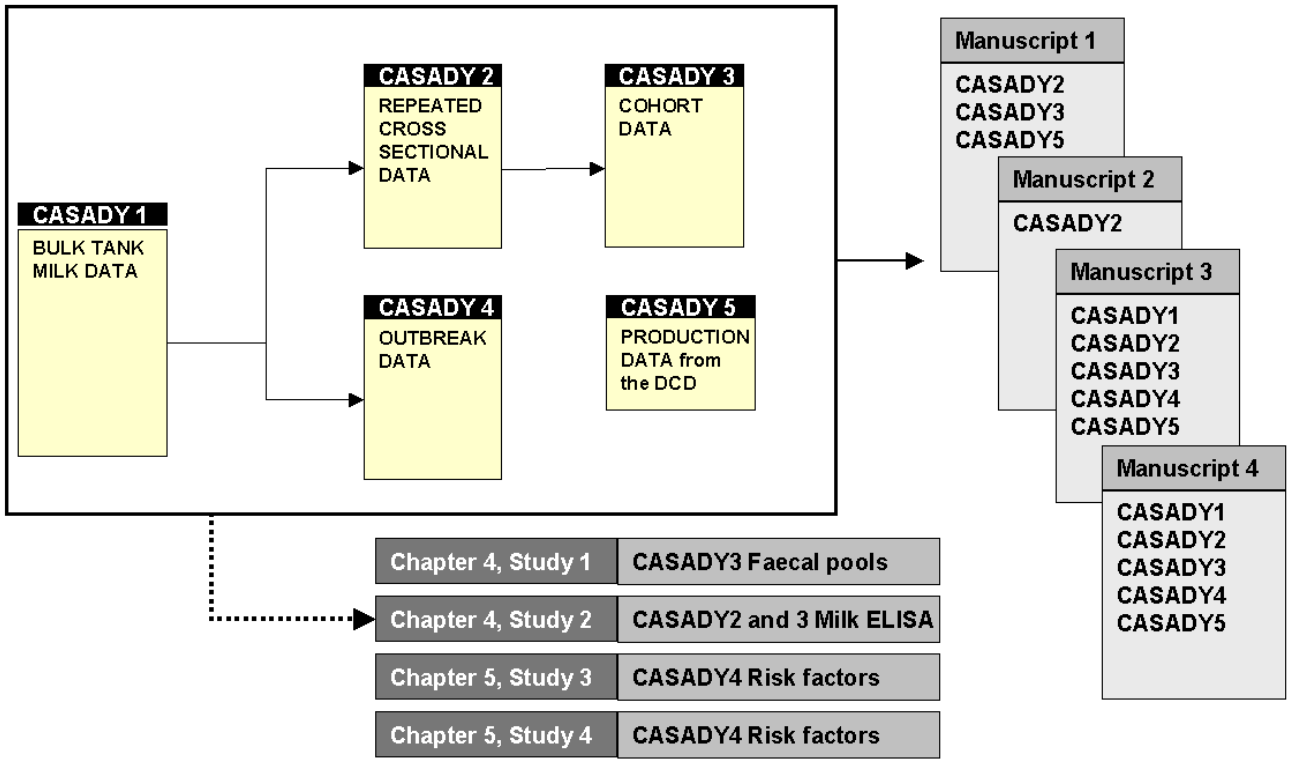


Figure 3.14 An overview of which datasets from CASADY were used in Manuscripts 1 through 4 and the 4 studies in Chapter 4 and 5.

Chapter 4. Interpretation of laboratory diagnostic methods for *Salmonella* Dublin infection in cattle

Validation of diagnostic tests involves a wide range of procedures, including clear definitions of test purposes and test protocols (based on finalized laboratory test optimisation and test standardisation), appropriate choice of validation methods and reference tests, selection of reference populations and subpopulations, sampling procedures, independent evaluation of test results, calculation, presentation and critical evaluation of relevant parameters[38].

In performance assessment of diagnostic tests there are several issues that need to be considered. The test used should ideally be 100% sensitive and 100% specific for detection of e.g. infection by the infectious organism in question. In this way, all tested and infected animals in the study population would be detected and no unspecific reactions, such as detection of cross-reacting antibodies, would occur. Such perfect tests are often referred to as gold standards. In reality, perfect gold standards rarely exist, and therefore less than perfect tests are frequently used as gold standards. This leads to possibilities for biased estimates of validity when new tests are compared to known imperfect tests[25].

Analytical sensitivity concerns the number of analytes required to trigger a positive test result (e.g. number of bacteria that must be present in a sample in order for the bacteriological culture method to detect its presence)[85]. Thus, the analytical sensitivity regards mainly the technical aspects of the test, which may be improved at the laboratory. The diagnostic sensitivity is the probability that a truly infected animal will be classified positive using the test[68]. The diagnostic sensitivity is influenced by the analytical sensitivity, the pathogenesis of the infectious organism in the tested animals and the laboratory validity of the test, mainly measured as repeatability and reproducibility of the test.

The analytical specificity is the ability of a diagnostic test to correctly identify the pathogen and distinguish it from other similar pathogens. For available diagnostics of *Salmonella* Dublin this is mainly an issue for the ELISAs, because sera or milk from animals infected with different salmonella serotypes may cross-react with antigens from *Salmonella* Dublin. This is often the case for *Salmonella enterica* serogroups B and D, which share the O-antigens 1 and 12[62,103]. The diagnostic specificity of a test is the probability that a truly non-infected animal will be classified negative using the test[68]. The diagnostic specificity of the

ELISA is therefore very much influenced by the analytical specificity. The pathogenesis, the activity of memory T-cells in the host and the half-life of immunoglobulins play an important role for the diagnostic specificity of previously infected animals that have cleared the infection[51]. Figure 4.1 illustrates examples of cross-reaction in the *Salmonella* Dublin milk ELISA from two cows known to be infected with *Salmonella* Typhimurium. The cows were infected in two herds where no *Salmonella* Dublin bacteria were found during the study period from September 2000 to June 2001. Similar cross-reactions can be found in bulk tank milk ELISA.

Konrad et al. (1994)[62] showed that it was possible to produce a pure O9 group-D antigen through a series of chemical procedures that eventually cleaved the lipopolysaccharides comprising O antigens 1 and 12, leaving a pure O9 ELISA antigen. When this ELISA was used on samples from animals experimentally infected with different group B salmonella serotypes, the cross reactions were reduced relative to the ELISA based on the antigen with O1, O9 and O12. This gave a significantly increased specificity of the indirect *Salmonella* Dublin ELISA.

The sensitivity and specificity estimates comprise important knowledge in the use of diagnostic tests, but often the predictive values of the test are more easily understandable to the people requesting a test result. The positive predictive value is the probability that the animal is truly infected when the test result is positive. The negative predictive value is the probability that the animal is truly not infected when the test result is negative. The predictive values are, however, strongly influenced by the prevalence of the infection in the investigated population. Sometimes likelihood ratios are used as an alternative to predictive values in an attempt to control for the prevalence[85].

In the remainder of this chapter, test validity and interpretation of test results from the bacteriological culture test for faecal samples and individual serum and milk ELISAs will be evaluated. Reasons for lack of validity or low predictive values will be discussed. An analysis of variance of factors affecting the bulk tank milk ELISA response against *Salmonella* Dublin using repeated measurements is also discussed. Manuscripts 1 to 3 contain most of the results and will be referred to whenever relevant.

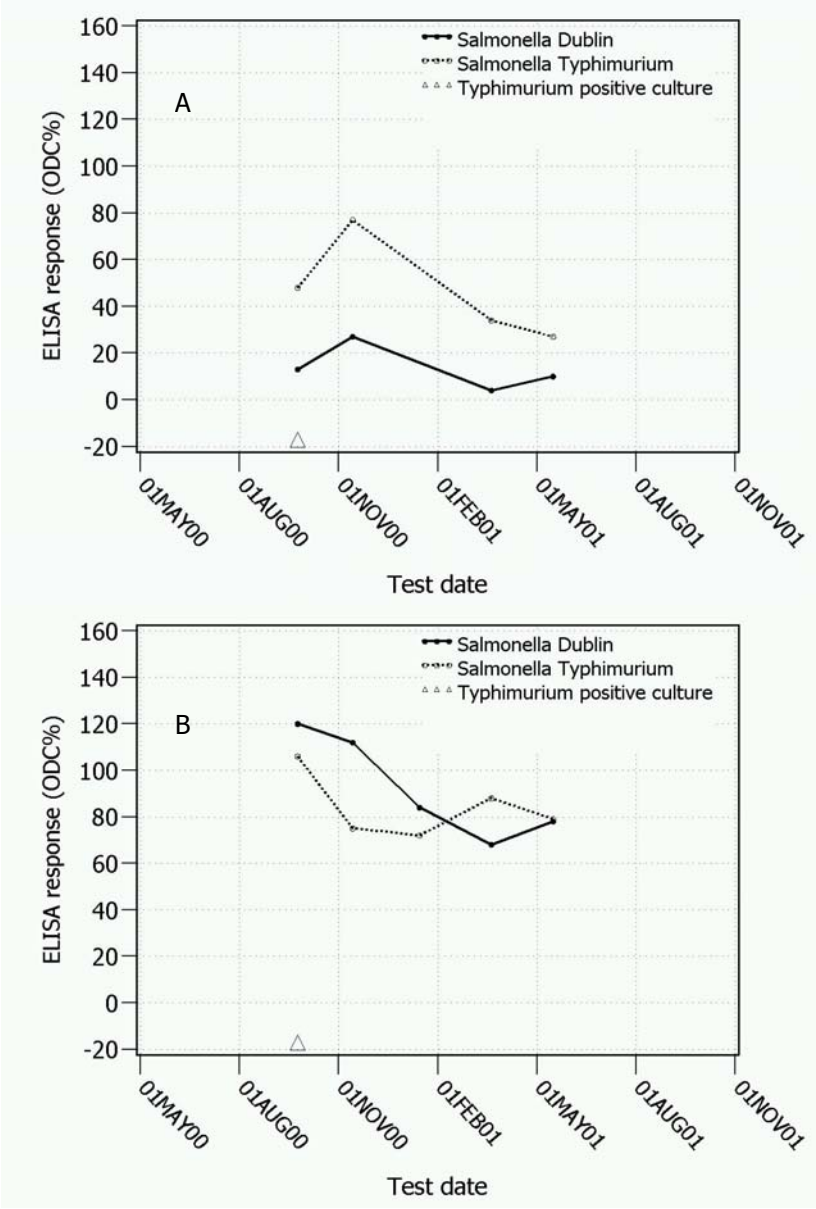


Figure 4.1 Examples of cross-reaction from *Salmonella* Typhimurium-specific immunoglobulins directed against O-antigens that are used in the *Salmonella* Dublin LPS-antigen used in the *Salmonella* Dublin milk ELISA. Milk from cow A has a low concentration of cross-reacting immunoglobulins. Milk from cow B has a high concentration of cross-reacting immunoglobulins. Both cows became infected with *Salmonella* Typhimurium during a severe disease outbreak in herd no. 14.

Two test validation methods were used: Classic and latent class estimation methods. Classic test validation is the most traditionally used method, where the sensitivity of a test is determined based on a set of samples from animals known to be infected. Either the animals may show pathognomonic symptoms, the pathogen may have been isolated from the animal, or the animal may have been experimentally infected with a dose known to cause infection in the host. The sensitivity of the test being validated is the proportion of test positives of all infected animals or animals with symptoms. The specificity is determined using a set of samples from known uninfected animals, and it is calculated as the proportion of test negatives of all uninfected animals. The drawback of classic test validation is that it may be difficult to find animals that can be correctly classified without introducing selection bias leading to an over- or an underestimation of the test validity.

In order to avoid misclassification errors or selection bias introduced by the use of an imperfect reference test, latent class test validation methods have become more widely used[25,84]. One such method is maximum likelihood estimation of test validity and prevalence. The method, which was initially described by Hui and Walter (1980)[55], uses two or more tests with unknown sensitivity and specificity in two or more populations with different prevalence of infection. In short, the principle is that given the data it is possible to estimate the most likely sensitivities and specificities of two or more tests in two or more populations if (i) the sensitivity and specificity of each test are the same in all populations, (ii) two of the tests are conditionally independent (i.e. the test validity of one test should not influence the test validity of the other test), and (iii) the prevalence in the populations vary. Different computational algorithms are available for this purpose. The latent class test validation methods may be more appropriate for validation of diagnostic tests for *Salmonella* Dublin than classic test validation methods, because the pathogen frequently leads to a latent infection that is difficult to detect, and because there is no true gold standard available for this infection, primarily due to the complicated pathogenesis. An advantage of the latent class validation method is that the validities of both tests are obtained. This gives an opportunity to quantify the validity of faecal culture tests for detection of *Salmonella* Dublin infected animals. The drawback of the latent class methods is that it is difficult to assess exactly for which stage of infection the method is estimating test validity. Also, the sample size required for estimation is larger than for classic test evaluation. However, the estimates are unbiased. For *Salmonella* Dublin, a comparison of the results from both validity estimation methods may lead to a better understanding of the test validity.

4.1 Bacteriological culture methods

As described in more detail in Chapter 2, section 2.3, the pathogenesis of *Salmonella* Dublin usually implies periods of time where the animal is infected, but no excretion of bacteria occurs. Therefore, bacteriological culture tests are not perfect with regard to sensitivity for detection of *Salmonella* Dublin infected animals. The analytical sensitivity for bacteriological culture methods for *Salmonella* Dublin is generally assumed to be good, though it varies with for instance choice of enrichment media[54,74]. Laboratory experiments at the Danish Veterinary Institute (DVI) have shown that in general less than 10 salmonella colony forming units (CFU) in a 22 g sample of swine faeces can be detected by the test procedure described in section 3.3.1.[57]. However, some difference in detection limits may be found between types of salmonella bacteria and between types of faeces, and it has not yet been ruled out that *Salmonella* Dublin could be more difficult to detect in cow faeces than other salmonella types. Thus, *Salmonella* Dublin may have a poorer analytical sensitivity than other types of salmonella, and the detection limits in cow faeces may be higher, because of factors such as structure of the faecal matter and competing ruminal microflora.

Usually, the lack of diagnostic sensitivity for *Salmonella* Dublin is considered to be due to the pathogenesis of the infection. The sensitivity of the bacteriological culture tests are known to be best for recently infected animals (1-15 days post-infection), untreated, diseased animals and carrier cows during the peripartum period where shedding is most likely to occur due to stress following for instance hormonal changes, release of corticosteroids into the blood stream and negative energy balance[20,54]. Wray and Davies (2000)[137] states that up to 50% of infected calves may be negative in faecal swabs. House et al. (1993)[54] found 3.35% of 985 faecal samples from 8 known *Salmonella* Dublin carrier cows and 17.26% of 643 faecal samples from 5 known carrier calves culture positive during a 12 month study period, where the animals were sampled several times a week. Although very informative with regard to diagnostic sensitivity of faecal culture in carrier animals, the number of animals used for that study was small. The diagnostic sensitivity of faecal bacteriological culture has not yet been properly estimated for detection of all stages of infected cattle.

Hypothesis 1:

The sensitivity of faecal bacteriological culture of Salmonella Dublin in cattle is age-dependent and can be estimated using latent class test validation.

A study was performed to estimate the sensitivity of the faecal culture method and the sensitivity and specificity of the serum ELISA in three different age groups. The study is described in Manuscript 2, "Validation of an indirect serum ELISA and a bacteriological faecal culture test for diagnosis of *Salmonella* serotype Dublin in cattle using latent class models". The results showed that the specificity was very close to 100% as would be expected for a bacteriological culture method with subsequent serotyping. For instance, if the bacteria is found in the faecal sample it had to be present in the animal unless cross contamination at the laboratory or at sampling had occurred. In Table 1 in Manuscript 2 the sensitivity estimates are illustrated for the faecal culture method together with the results of the estimates for the serum ELISA in the three age groups 0-99 days of age, 100-299 days of age and ≥300 days. The sensitivity was found to be between 6 and 14%, and though there was a tendency for lower sensitivity for the oldest age group no statistically significant difference could be detected between the age groups. The test procedure validated in this study was the pool-first method where faecal matter from 5 animals was pooled into one. Only positive pools were retested on an individual basis.

The latent class method used was able to provide estimates for the sensitivity of the faecal culture method. However, indications were found that the assumptions for the method were not strictly adhered to, which may have lead to estimation errors. It was found that the sensitivity of the test and the prevalences estimated in the test populations were not independent. One of the assumptions of the latent class method is that the validity of the test should be equal in the test populations. If the validity was found to be related to the prevalence, this was not the case, because another assumption for the method is that the prevalences in the test populations are different.

The conclusion in relation to hypothesis 1 is that a rough estimate of the sensitivity of the pool-first faecal culture method for detection of potential shedders of *Salmonella* Dublin could be obtained by a latent class method using maximum likelihood estimation. No obvious difference in test performance between age groups of cattle was detected.

4.2 Relative validity of faecal pools compared to individual samples (Study 1)

In Manuscript 2 it was shown that the sensitivity of the pool-first faecal culture method for detection of potential shedders of *Salmonella* Dublin was fairly poor. The method of pooling of cattle faecal samples for detection of *Salmonella* Dublin has not been validated. One study of detection of *Salmonella enterica* in swine faeces showed that the weight of the faecal sample significantly influenced the sensitivity of the test[32]. The best sensitivity was found in samples of 25 g compared to 1 g and 10 g of faecal material. In another study of swine faeces it was found that homogenisation of the faecal sample before taking a subsample for pre-enrichment increased the sensitivity[16]. The less material taken out for pre-enrichment, the more important was homogenisation. The study by Cannon and Nicholls (2002) showed the best performance for 25 g and 50 g of faecal matter per sample as compared to 1 g and 10 g. This is likely to be due to clustering of bacterial organisms in the sample. Clustering of salmonella bacteria may also be a problem in faeces from cattle.

In the pool-first method used in this thesis, 5 g of faecal matter from each animal was used. The procedure did not include homogenisation of the faecal sample before the pre-enrichment step. Thus, a possibility for a reduced sensitivity of the pool-first test procedure compared to the individual faecal culture test procedure using 25 g of faecal material is present.

Hypothesis 2:

Pooling of faecal samples reduces the sensitivity of the bacteriological faecal culture method for detection of Salmonella Dublin bacteria.

A study was performed to investigate the performance of the pool-first method compared to the individual faecal culture method. The samples were mainly collected as part of sampling activity 3. For each faecal sample collected, 5 g were taken and pooled with 5 g from each of four other faecal samples. Pre-enrichment in peptone buffered saline lead to homogenisation of the pooled sample. Another 25 g (or the rest of the sample available) of each of the five faecal samples were used directly in the pre-enrichment step without pooling. Table 4.1 shows a two-by-two table of the laboratory results from 536 pools.

Table 4.1 Laboratory results from individual and pooled faecal samples

	Individual FC results[a]		
	FC +	FC –	Total
Pool +	19	2	21
Pool –	25	490	515
Total	44	492	536

[a] FC=Faecal culture

FC+ indicates that at least one individual sample was culture positive for *Salmonella* Dublin in the pool

FC– indicates that no individual samples from the animals in the pool were culture positive

A total of 19 pools were culture positive out of 44 that should have been positive according to the individual sample results, giving a relative sensitivity of 43% (95%CI: 28-59%) of the pool-first method compared to the individual faecal culture method. A total of 490 pools were culture negative out of 492 that should be negative according to the individual sample results, giving a relative specificity of the pool-first method compared to the individual faecal culture method of 99.6% (95%CI: 98.5-100%). The results should not be confused with overall validity estimates, which were estimated in section 4.1 for the pool-first test procedure. The relative sensitivity of the pool-first test procedure to the direct individual test procedure indicated that using the pool-first test procedure reduces the sensitivity of the bacteriological faecal culture test to 43% of the sensitivity of the individual faecal culture test procedure. The relative specificity of the pool-first test procedure indicates that the individual faecal culture test procedure is also not perfect with regard to sensitivity; if a pool was positive, at least one individual sample ought to have been positive as well. The main weakness of the present study of pool validity, is that the sensitivity of the individual faecal culture test procedure is not perfect. Assuming that pooling of faecal samples reduces the sensitivity of the faecal culture method by 57%, and assuming that the overall diagnostic sensitivity of the first-pool test procedure is between 6% and 14% as estimated in Manuscript 2, an estimate of the sensitivity of the individual faecal culture test procedure is 14% to 32%. Therefore, it cannot really be used as a gold standard to which the pool-first test procedure can be compared. Supporting the findings are the two studies of swine faeces referred to in the beginning of this section[16,32] and the study by House et al. (1993)[54]. The reasons for the reduced sensitivity of the pool-first test procedure compared to the direct individual culture test procedure may be lack of homogeneity of the sample and the amount

of faecal matter collected and used for pre-enrichment, however more work needs to be done to determine the optimal faecal culture procedure and detection limits for *Salmonella* Dublin in cattle faeces.

4.3 ELISAs for individual diagnosis

In section 2.3 it was described how the specific immune response becomes activated when animals are invaded by *Salmonella* Dublin bacteria. Currently, no routinely used tests are available to detect the activation of the cell-mediated immunity against *Salmonella* Dublin, but immunoglobulin levels (indicating a humoral immune response) in blood and milk can be measured by the use of for instance the indirect ELISAs described in section 3.3.2. The methods may vary slightly between laboratories. ELISAs have poor sensitivity early in the infection period, because the humoral immune response is delayed in relation to the time of initial infection. Figure 4.2 illustrates a 1-2 week delay in the humoral immune response in a cow and a 7-8 week delay in a calf infected at birth. This calf is particularly illustrative because it appears from the graph that it did not receive salmonella specific antibodies through colostrum. This would be the case when colostrum comes from uninfected mothers. The total immunoglobulin concentration measured from this calf at 1 day of age was 69 mg/ml indicating that colostrum derived immunoglobulins had been acquired by the calf. Therefore, the lack of specific salmonella antibodies was not due to lack of colostrum uptake. The measurement of total immunoglobulin was performed as a routine diagnostic test (sandwich ELISA) at DVI. The calf had acute salmonellosis with typical symptoms (bloody diarrhoea, fever, lack of appetite and recumbency) throughout the first week of its life, but recovered with antibiotic and fluid therapy. The stress of clinical disease in this calf may be one of the reasons the humoral immune response was very delayed. However, young calves have been shown to have very little immunoglobulin production against *Salmonella* Dublin infection the first 11-12 weeks of their lives even when they are not clinically ill[21].

A study on a large dairy herd in California showed that animals that remained culture positive in milk and faecal samples collected monthly over 6 months all became positive for IgG measured by a similar serum ELISA to the one used in this thesis. This indicates that the ELISA will eventually become positive if the animal is infected, i.e. the sensitivity of ELISA is good – especially for repeated sampling[54]. After 2-3 weeks post-infection, the ELISA may in fact be more sensitive than bacteriological culture methods for detection of

Salmonella Dublin infected animals. Also, a positive correlation was found between shedding of *Salmonella* Dublin and the antibody titre measured, suggesting that more frequent shedding leads to higher antibody levels in blood and milk[54].

A similar ELISA was evaluated with regard to validity for detection of newly infected and carrier animals in two studies. Veling et al. (2000)[121] found that the O-antigen based LPS serum ELISA for IgG detection had a sensitivity of 32.0% at a cut-off titre value of 100, when validated against positive faecal shedding of bacteria in 50 infected animals soon after a new outbreak of *Salmonella* Dublin in 13 Dutch herds. The specificity was evaluated to be 99.3%, using 840 serum samples from herds without any history of salmonella infection on the premises, and where all animals tested faecal culture negative. The test validity was also determined for known active carrier animals. In that study, active carrier animals were defined as animals with at least three successive *Salmonella* Dublin positive faecal cultures and a sampling interval of at least 14 days. The sensitivity was estimated to be 94.4% based on a sample size of 18 active carrier animals. The ELISA[121] was performed slightly different from the ELISA used in the present thesis. For instance, the conjugate used in the paper by Veling et al. (2000)[121] to detect bound antibodies was a monoclonal antibody against IgG_1, whereas the conjugate used in the ELISA used in the present thesis contained antibodies against both heavy and light chains of IgG. This may lead to differences in specificity and sensitivity of the tests, because several classes of immunoglobulins (e.g. IgM, IgG_1 and IgG_2) may in fact be bound by the conjugate of the ELISA used here. Further, the titre values are not directly comparable to ODC%-values.

In a study by Spier et al. (1990)[108] the purpose was to evaluate the use of an indirect serum ELISA for identification of carrier animals, and to differentiate carriers from cows given bacterin and cows with experimentally induced *Salmonella* Dublin infections that did not become carriers. The numbers of animals used in that study were limited to a total of (i) 6 carriers with repeated culture positive faecal and milk samples over a period of 1 year and *Salmonella* Dublin positive cultures at necropsy, (ii) 7 experimentally infected cows that ceased shedding of bacteria and did not have salmonella bacteria found at necropsy, and (iii) 7 negative control cows. The authors concluded that two positive serum IgG titres to *Salmonella* Dublin obtained 2 months apart was a better predictor of carriers than two serum samples obtained 1 months apart, single samples or IgG:IgM ratios. Also serum IgG titres gave more consistent results than milk IgG titres. A later study from the same research group, however, suggested that the optimal test scenario for the use of serum IgG titres for carrier

prediction was 3 samples obtained over a period of 120 days. This was suggested for discrimination between carriers and transiently infected animals[104].

As described above, the indirect serum ELISA for detection of IgG against *Salmonella* Dublin has been acknowledged in the literature as a fairly sensitive diagnostic tests for *Salmonella* Dublin infection in active carrier cattle. However, the sensitivity of ELISAs for infected, but non-shedding or periodically shedding animals that do not become detected due to the lack in sensitivity of the bacteriological culture methods, still remains to be evaluated. Also, more test evaluation of the similar test used in individual milk samples is required. This is the basis for the studies of individual serum and milk ELISA validity and interpretation below.

Hypothesis 3:

The validity of the indirect serum ELISA for detection of Salmonella Dublin infection in cattle is age dependent

Hypothesis 4:

The sensitivity of individual ELISAs are better than the sensitivity of faecal culture methods for detection of Salmonella Dublin infection in cattle

Hypothesis 5:

Unbiased sensitivity and specificity estimates of individual ELISAs can be obtained using latent class analysis as opposed to using classic test validation methods.

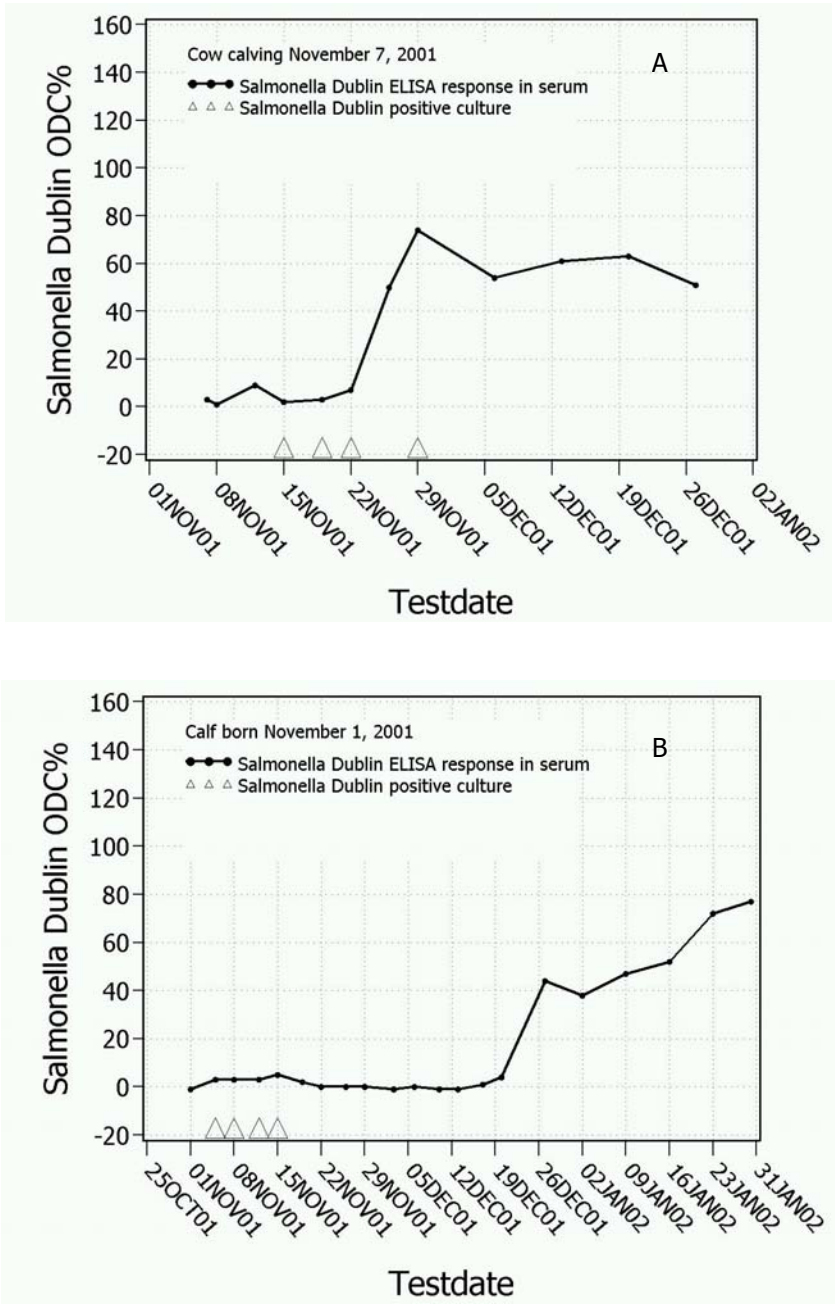


Figure 4.2 Examples from CASADY3 of *Salmonella* Dublin specific humoral immune response after initial infected in A: a cow, B: a newborn calf.

4.3.1 Validity estimation of the serum ELISA

In Manuscripts 1 and 2 the serum ELISA was validated for three different age groups:

1: 0-99 days old
2: 100-299 days old
3: ≥ 300 days old

In Manuscript 1 the test was validated using a classic test validation method where all culture negative animals came from assumed uninfected herds. In other words, all animals from *Salmonella* Dublin infected herds that were not shedding bacteria in faecal samples on the day of serum sample collection were not included in the analysis. This may lead to biased results. The results were displayed as ROC curves and plots of sensitivity and specificity with 95% confidence limits; see Figures 2 and 3 in Manuscript 1. The curves can be used to read the sensitivity and specificity at different cut-off values. The area under curve (AUC) of ROC curves were used as an overall measure of the performance of the test. Comparison of the AUCs for the three different age groups showed statistically significant difference in overall test performance between all age groups with the best performance being for calves and young stock aged 100-299 days, and the poorest performance being for young calves, aged 0-99 days. This finding was not surprising, because it is known that passively transferred maternal antibodies may give false positive reactions in the ELISA in young calves, and because it is known that calves up to 12 weeks of age have much more delayed antibody production compared to older cattle[21,96].

In Manuscript 2 sensitivity and specificity of the serum ELISA were estimated together with the sensitivity of the pool-first faecal culture test using a latent class method. The population from CASADY2 (except herds with only *Salmonella* Typhimurium isolations) was divided into 2 populations based on veterinary practices. The estimation was performed using maximum likelihood estimation. Results were reported at two cut-off values in the ELISA, 25 ODC% and 50 ODC% which are currently used in the National Surveillance Program for *Salmonella* Dublin. ROC-curves were presented for the serum ELISA. In Figure 4.3 the ROC-curves from the classic method and the latent class method are presented together for each age group for comparison. In Figure 4.4 the plots of sensitivity and specificity for a wide range of cut-off values illustrate the difference in sensitivity and specificity estimates for the two methods.

The conclusions in relation to hypotheses 3 to 5 are that differences between the three age groups in overall performance of the serum ELISA was found using both methods as illustrated in Figures 4.3 and 4.4. The test appears to have the highest validity for calves and young stock between 100 days and 300 days of age. However, the results suggest that the specificity is overestimated by the classic validation method, and the sensitivity may in fact be slightly underestimated by the classic validation method – especially for the oldest age group. This is likely to be due to selection bias related to the sampling strategy in the classic method, where not all infection stages in the test populations are represented. The sensitivity and specificity of ELISAs greatly depend on the selected cut-off value. At cut-off 50 (which is currently used in the Danish National Surveillance Program) the sensitivity is approximately 21% for calves aged 0-99 days, approximately 74% for young stock aged 100-299 days and between 45% and 59% for cattle ≥300 days of age, depending on which evaluation method is used. The specificity at cut-off 50 is between 96% and 99% for calves aged 0-99 days, between 96% and 100% for young stock aged 100-299 days and between 89% and 98% for cattle ≥300 days of age. Choosing different cut-off values for the different age groups can be used to optimise the validity of the serum ELISA.

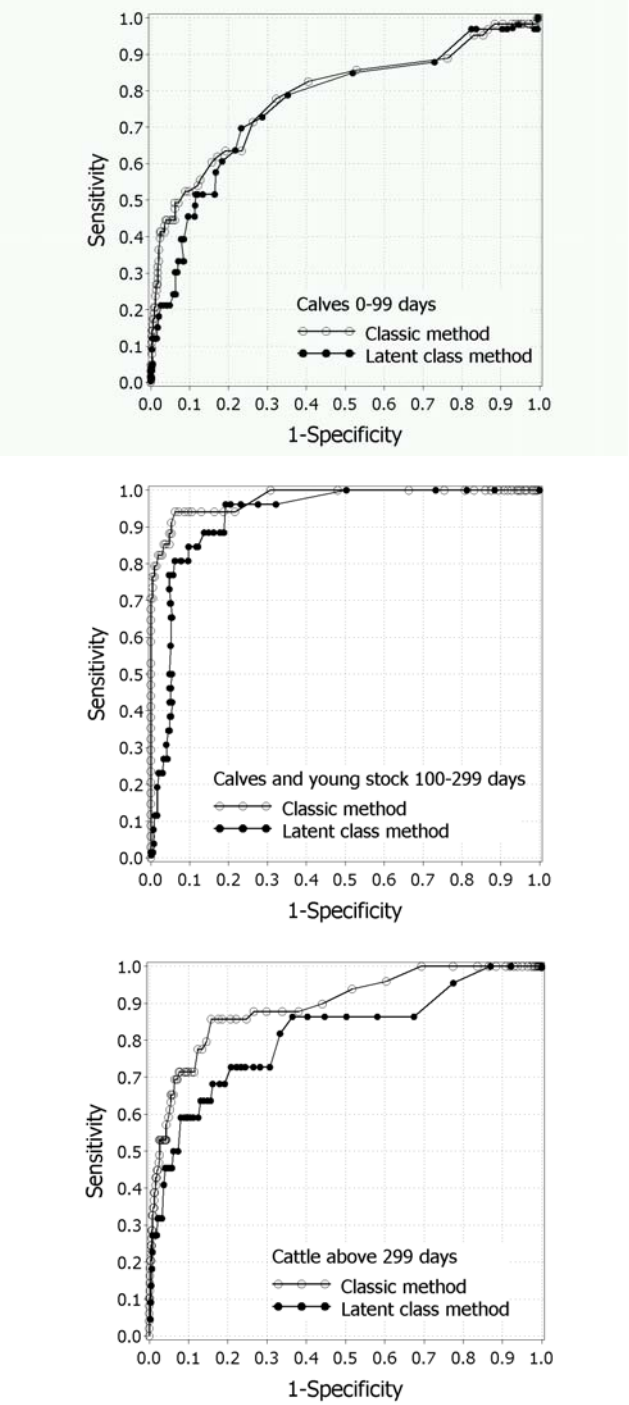


Figure 4.3 Receiver operating characteristics (ROC) curves for an indirect serum ELISA for detection of *Salmonella* Dublin infection in cattle in three different age groups.

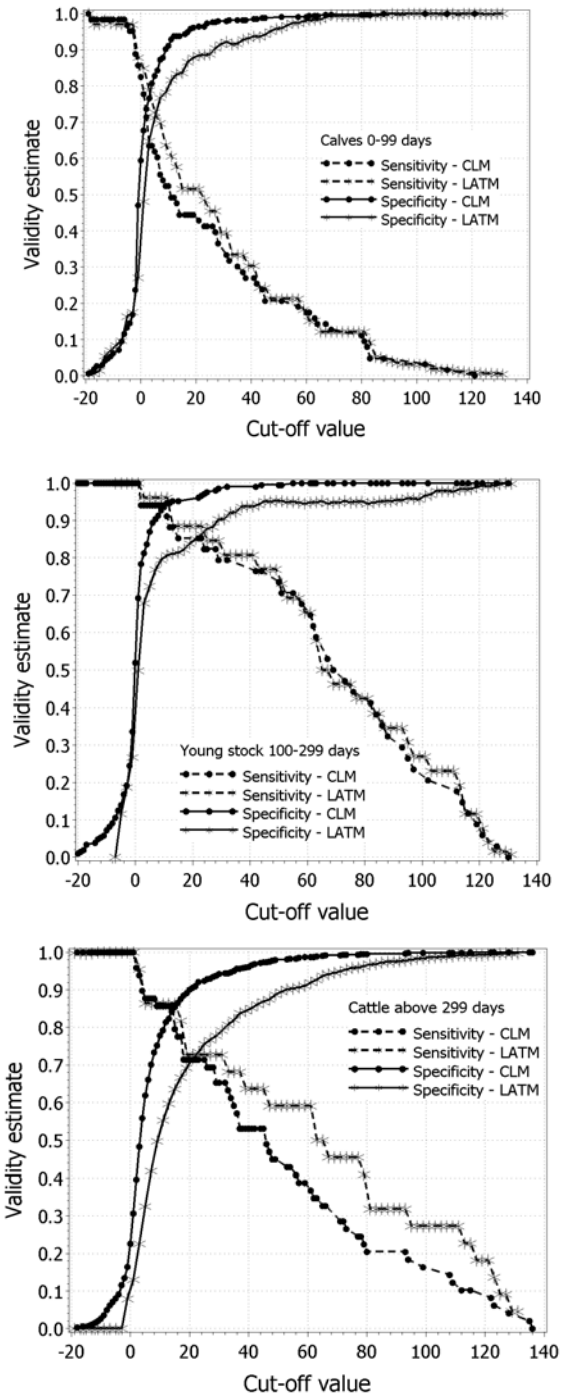


Figure 4.4 Sensitivity and specificity of an indirect *Salmonella* Dublin ELISA in three age groups estimated by a classic test validation method (CLM) and a latent class method (LATM).

4.3.2 Validity estimation of the individual milk ELISA (Study 2)

The individual milk ELISA was validated using the same methods as described for the serum ELISA. All lactating animals were evaluated as one age group. Samples that were collected less than 7 days after calving were disregarded due to the known risk of false positive reactions from colostrum.

For the classic test evaluation method, one sample pair (one milk sample and one faecal sample) of all cows from herds that were classified salmonella negative according to the sampling results in sampling activity 2, were used as the negative population used for specificity calculations. All in all 752 negative sample pairs from 9 herds and 13 faecal positive sample pairs from 4 herds with *Salmonella* Dublin faecal culture positive cows were included in the study. One sample pair per cow was selected by random selection.

For the latent class test evaluation method, a total of 2893 cows from 27 herds were divided into 2 populations based on veterinary practices, and the estimations of sensitivity, specificity and population prevalences were calculated as described in Manuscript 2 for the serum ELISA and faecal culture method. Only 35 of these cows were culture positive on the same day the milk sample was collected. This may pose a problem in the estimation of sensitivity for the faecal culture test. In Figure 4.5 the sensitivity and specificity estimate results of the classic and the latent test evaluation of the milk ELISA are shown together with estimates of sensitivity of the faecal culture method from the latent class test evaluation. In Figure 4.6 the ROC curves for the individual milk ELISA calculated by the classic and latent class estimation methods are shown. Statistical comparison of the AUCs from these two curves is not possible, as the underlying data for sensitivity and specificity at each cut-off value are not available from the latent class analysis.

At cut-off 25 ODC% the milk ELISA has an estimated sensitivity of 77-78% and an estimated specificity of 65-86% depending on which test evaluation method is used. At cut-off 50 ODC% these estimates were 42-43% and 81-94%, respectively. In relation to hypotheses 3 to 5 it can be concluded that the milk ELISA has a similar sensitivity and a slightly lower specificity than the serum ELISA at cut-off 50 ODC% when compared to the oldest age group evaluated for the serum ELISA. At cut-off 25 ODC% the two tests are similar in validity. The sensitivities of both ELISAs are better than the faecal culture method, but a risk of false positive results is present and should be accounted for when using ELISA for prevalence estimation and other epidemiological studies. The specificities of the ELISAs are

generally poorer for animals above 300 days of age than for calves and young stock below the age of 300 days.

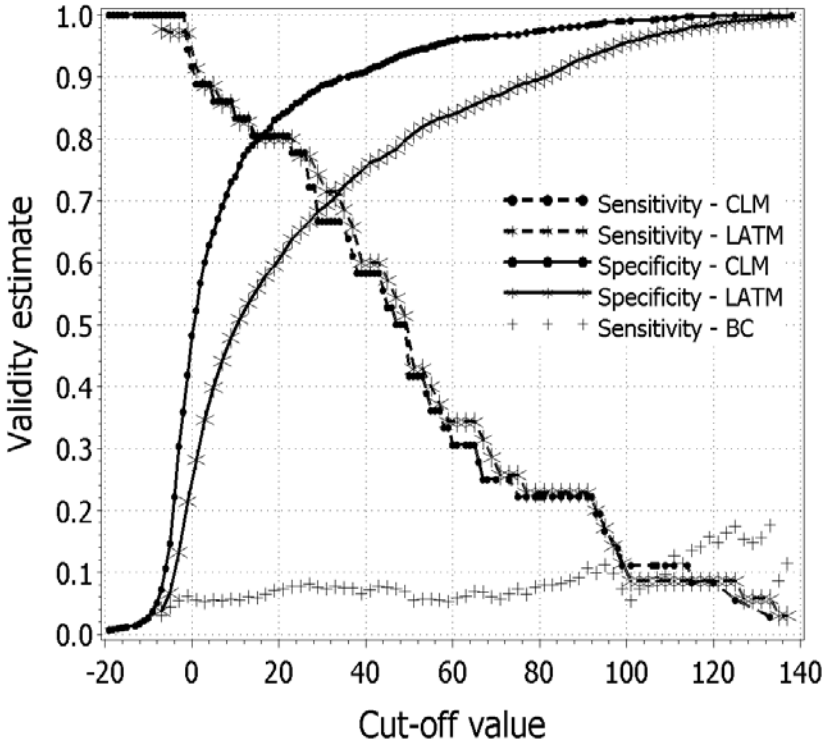


Figure 4.5 Validity estimates for the milk ELISA and faecal culture method 1 calculated by maximum likelihood estimation (latent class test validation method) in two populations based on veterinary practices from CASADY2 and CASADY3.

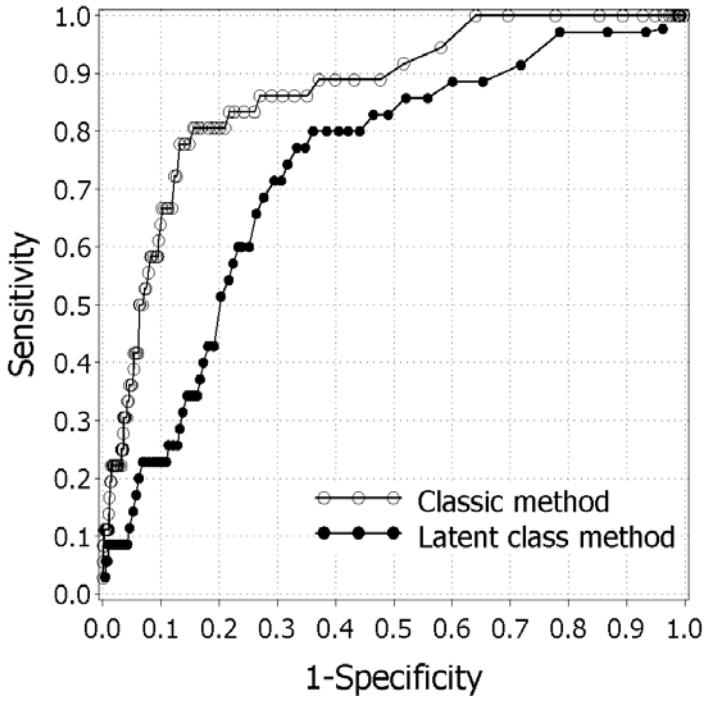


Figure 4.6 ROC curves for the individual milk ELISA evaluated by classic and latent class methods.

4.4 Interpretation of bulk tank milk ELISA response

Bulk tank milk has been used routinely for surveillance of several infections in cattle (i.e. BVD, IBR)[120]. The Danish National Surveillance Program for *Salmonella* Dublin is likewise based on bulk tank milk ELISA response against *Salmonella* Dublin. This raises questions about the validity of this test and creates a need for more knowledge about factors accounting for variation in the bulk tank milk measurements.

As described in Chapter 3 bulk tank milk samples were collected routinely from 94 herds through the milk quality recording scheme 11 times per year from March 2000 to March 2003.

Hypothesis 6:

> *The bulk tank milk Salmonella Dublin ELISA response reflects the level of infection particularly in the lactating cows, and the presence of Salmonella Dublin infection in a herd*

Hypothesis 7:

> *The variation in bulk tank milk ELISA response can be explained by factors related to the lactating cows*

In Table 4.2 the types of salmonella isolations in relation to the *Salmonella* Dublin level appointed in the National Surveillance Program for the 35 dairy herds participating in CASADY2, CASADY3 and CASADY4 are shown. To further analyse factors contributing to variation in bulk tank milk ELISA response against *Salmonella* Dublin, the study described in Manuscript 4 was performed.

To illustrate the dynamics of the bulk tank milk ELISA response against *Salmonella* Dublin in relation to knowledge about the herds, serological and bacteriological findings of some of the 35 herds that participated in the activities related to this thesis are presented below. All the herds are listed by herd number in Appendix B.

<u>Herd number 4</u>

This herd was asked to participate because the first bulk tank milk measurements were high. It was a fairly small family driven dairy herd with approximately 50 lactating cows in a tie

stall. Management around calving, feeding of colostrum to calves and housing of neonatal calves was well controlled, and the calf barn was kept separate from other animals in the herd. The bull calves were kept for intensive rearing and were sold for slaughter at approximately 10 months of age. Approximately 50 bull calves and young stock were kept together in six pens until slaughter. The herd had an outbreak of *Salmonella* Dublin two years before sampling activity 2 was initiated, and had problems with diarrhoea amongst the calves of all ages. Antiserum treatment was used for neonatal calves in an attempt to reduce clinical symptoms in this group.

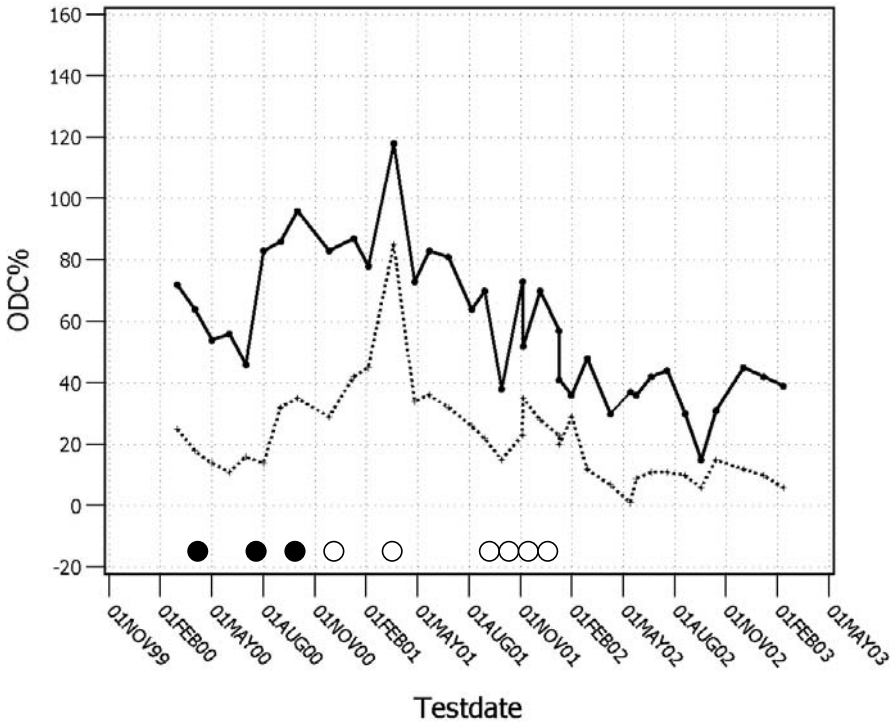


Figure 4.7 Bulk tank milk ELISA response against *Salmonella* Dublin (solid line) and *Salmonella* Typhimurium (dashed line) in herd number 4. ● indicates that *Salmonella* Dublin culture positive samples were found in the herd, ○ indicates that no bacteriological cultures were positive in the herd despite efforts to isolate the bacteria (sample collection activities number 2 and 3).

Salmonella Dublin was cultured from the herd on several occasions from April to October 2000 in:

- 5 calves from 2 to 11 months old
- 8 swabs in the calf and young stock barn section
- 1 dung pit sample

During the period the herd was studied, the disease symptoms related to *Salmonella* Dublin and bacteriological isolations ceased to occur. The bulk tank milk ELISA response against both *Salmonella* Dublin and *Salmonella* Typhimurium over time is shown in Figure 4.7.

Herd number 7

This herd was also asked to participate because the first bulk tank milk measurements were high. The herd was a large (for Danish standards), high producing dairy herd with approximately 110 lactating cows in a loose housing system. Management around calving was problematic in the sense that most calvings took place in a common calving area where 6-10 late term and fresh cows were kept together in too little space. Feeding of colostrum to calves did not have high priority, but was given when convenient. Housing of neonatal calves and calves up to 4-5 months was well organised in a fairly new calf house with large single pens and open air ventilation. The owner gave the information that *Salmonella* Dublin was introduced to the herd around 1995 with some infected calves that were bought from another herd, and caused an outbreak of disease among calves. At the first 3 visits in year 2000 no salmonella was isolated, but from February 2001 and throughout the year and the beginning of 2002, the following *Salmonella* Dublin isolations were made from the herd:

- 10 calves below 3 months of age
- 21 young stock and heifers up to first calving
- 8 adult cows
- 7 environmental swab from all barn sections
- 2 dung pit samples

Besides that, *Salmonella* Typhimurium, *Salmonella* Saintpaul and non-typable salmonella isolations were made in this herd. The herd experienced a disease outbreak among heifers and cows probably related to poorly stored ensilage around New Years 2000-2001. This

may have lead to a drop in immune capacity in the herd with renewed shedding from carriers and increase spread of salmonella in the herd. The bulk tank milk responses are shown in Figure 4.8.

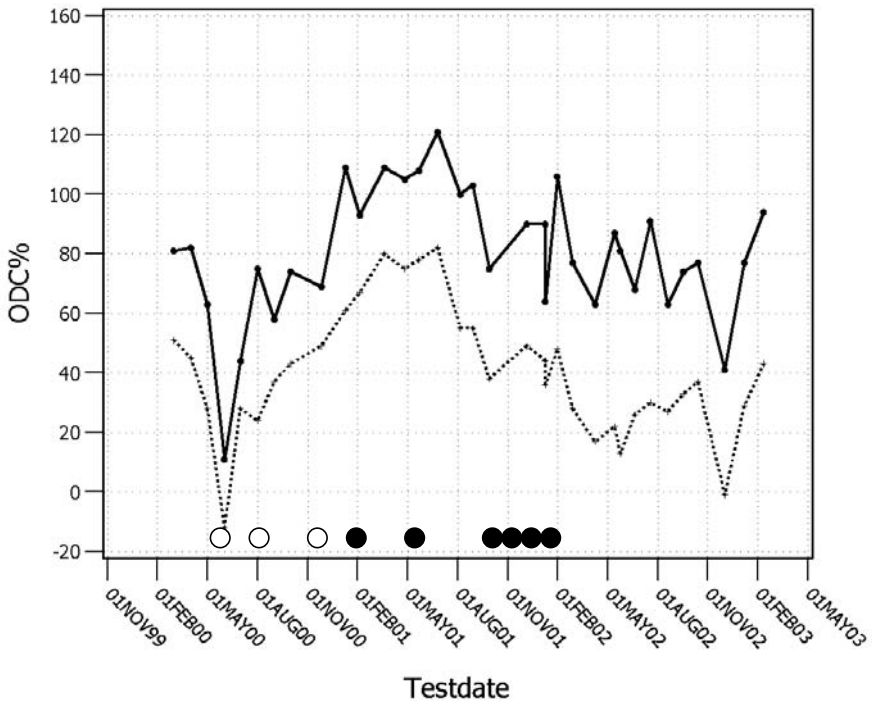


Figure 4.8 Bulk tank milk ELISA response against *Salmonella* Dublin (solid line) and *Salmonella* Typhimurium (dashed line) in herd number 7. ● indicate that *Salmonella* Dublin culture positive samples were found in the herd, ○ indicate that no bacteriological cultures were positive in the herd despite efforts to isolate the bacteria (sample collection activities number 2 and 3).

Herd number 18

This herd was asked to participate because it had low levels of antibodies against salmonella in the first bulk tank measures. It was expected that this herd could be used as a negative reference herd. The herd was initially a tie stall herd with 60 lactating cows, but in the beginning of 2001 the herd took a new large loose housing cow barn in use. The herd was visited in the Fall 2000 and 4 times in 2001 with approximately 3 months between each visit. No *Salmonella* Dublin or *Salmonella* Typhimurium was isolated from this herd, but in January 2001 the dung pit sample collected from the young stock barn was found positive for *Salmonella* Agona. In Figure 4.9 the bulk tank milk response from herd 18 is shown.

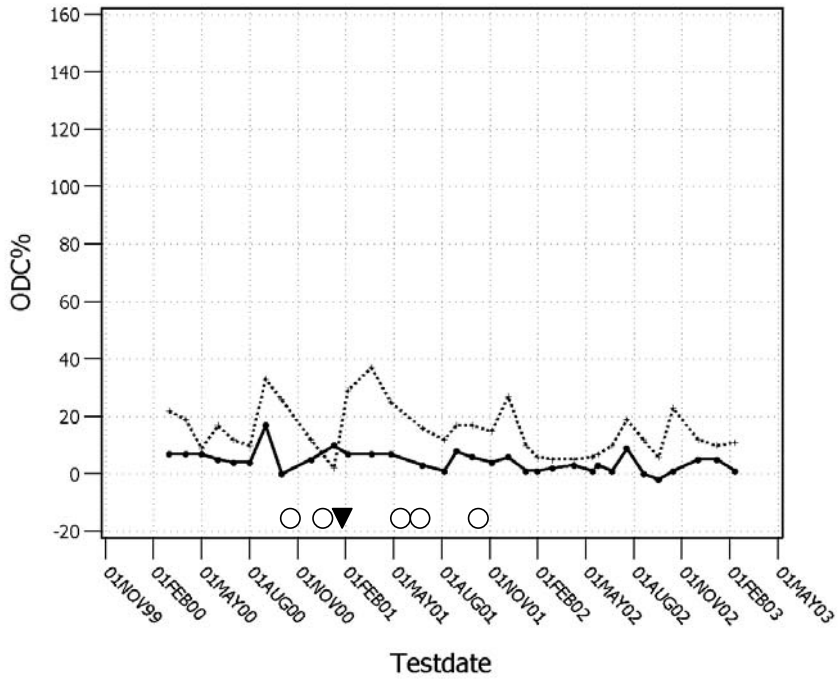


Figure 4.9 Bulk tank milk ELISA response against *Salmonella* Dublin (solid line) and *Salmonella* Typhimurium (dashed line) in herd number 18. ○ indicate that no bacteriological cultures were positive in the herd despite efforts to isolate salmonella bacteria (sample collection activity number 2). ▼ indicates that a dung pit sample was positive for *Salmonella* Agona.

Herd number 9

This herd was asked to participate because it had high levels of antibodies against *Salmonella* Dublin in the first two bulk tank milk measurements. The owners had bought the herd a few years before the project started and was in the middle of a process of expanding and rebuilding the barns. Initially the herd had approximately 115 lactating cows but this number increased to 140 during the study period. The barn system was loose housing. Young calves were kept in individual calf huts outside until the age of approximately 8 weeks. Young stock was kept in a barn with large common pens until they were moved into a loose housing barn section for heifers and dry cows. Clinical problems with diarrhoea in the young calves and pneumonia in the young stock was periodically seen throughout the study period. In Figure 4.10 the bulk tank milk response from herd 9 is shown. One cow was found to excrete *Salmonella* Typhimurium in one faecal sample.

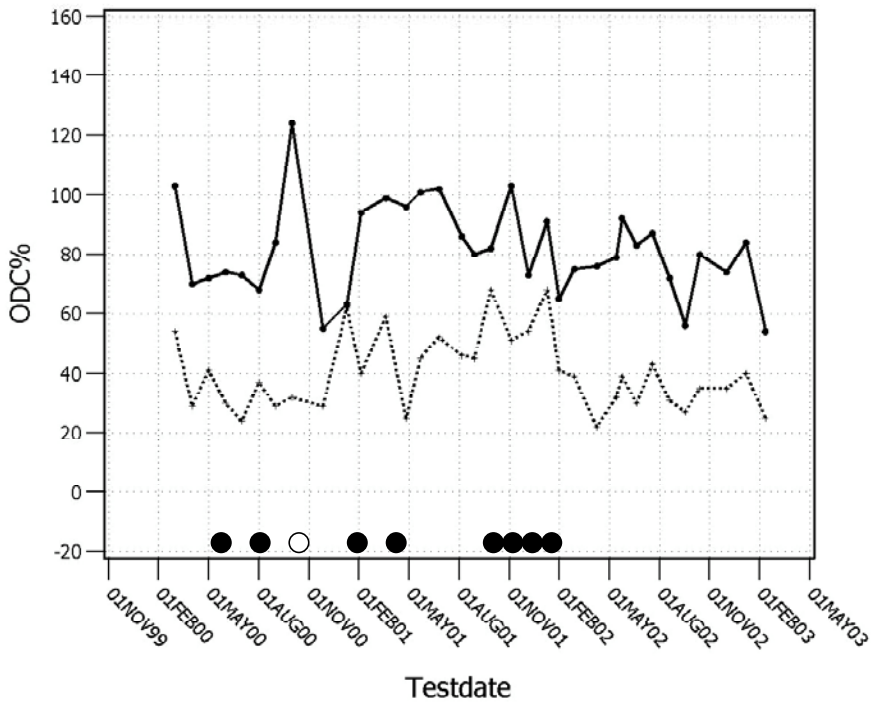


Figure 4.10 Bulk tank milk ELISA response against *Salmonella* Dublin (solid line) and *Salmonella* Typhimurium (dashed line) in herd number 9. ● indicates that *Salmonella* Dublin culture positive samples were found in the herd, ○ indicates that no bacteriological cultures were positive in the herd despite efforts to isolate the bacteria (sample collection activities number 2 and 3).

The following *Salmonella* Dublin isolations were made in the herd from May 2000 to January 2002:

- 18 samples from calves below 3 months of age
- 9 young stock and heifers up to first calving
- 4 adult cows
- 5 environmental swab from all barn sections

Herd number 11

The herd experienced a diagnosed *Salmonella* Dublin disease outbreak in May-June 2001, where three calves aged 9-14 days died. One heifer continued to excrete *Salmonella* Dublin

at consecutive visits and was eventually bought for research purposes in the Salmonella-microbiology project under the Kongeå-project. At autopsy in February 2002 *Salmonella* Dublin was isolated from the lung lymph nodes and the liver. The bulk tank milk response over time is shown in Figure 4.11. The following isolations of *Salmonella* Dublin were made in the herd:

- 21 samples from calves below 3 months of age
- 7 samples from young stock and heifers up to first calving
- 9 samples from adult cows

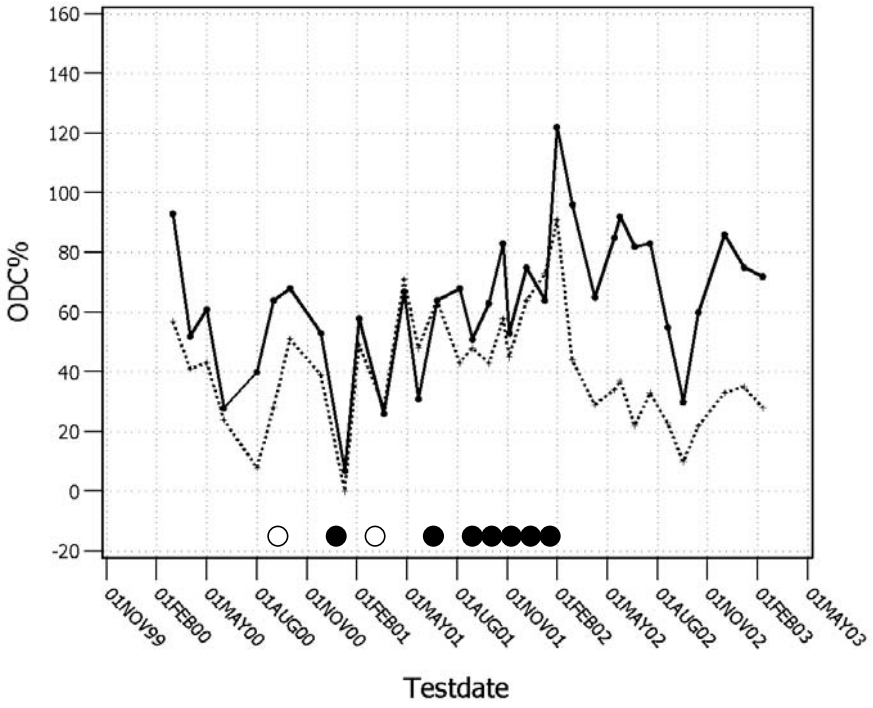


Figure 4.11 Bulk tank milk ELISA response against *Salmonella* Dublin (solid line) and *Salmonella* Typhimurium (dashed line) in herd number 11. ● indicates that *Salmonella* Dublin culture positive samples were found in the herd, ○ indicates that no bacteriological cultures were positive in the herd despite efforts to isolate the bacteria (sample collection activities number 2 and 3).

Herd number 23

This herd was asked to participate because of high *Salmonella* Dublin ELISA response in bulk tank milk in the first two measurements. The herd was a Jersey herd with approxi-

mately 75 lactating cows kept in tie stalls. Good management and high hygiene standards appeared to be of high priority in this herd. The young calves were kept in both single pens and common pens with 2-4 calves in separate barn section. All young stock (approximately 50 animals) was kept at a different barn 2 km away from the cows and calves. As shown in Figure 4.12 the bulk tank milk response against *Salmonella* Dublin was continuously medium to high throughout the study period. The herd was visited 5 times with 3-month intervals from November 2000 to November 2001 and all animals were sampled as described in Chapter 3. No salmonella bacteria were isolated from this herd.

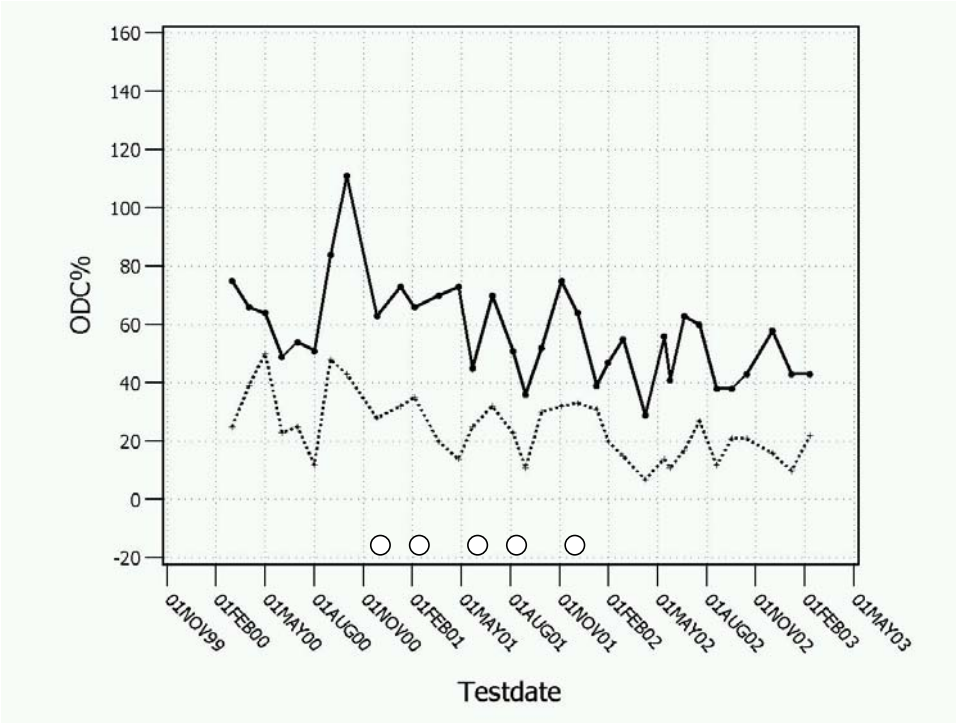


Figure 4.12 Bulk tank milk ELISA response against *Salmonella* Dublin (solid line) and *Salmonella* Typhimurium (dashed line) in herd number 23. ○ indicates that no bacteriological cultures were positive in the herd despite efforts to isolate salmonella bacteria (sample collection activity number 2).

In order to study the effect of the prevalence of infected animals in different age groups on the bulk tank milk ELISA response, a study of 31 selected sample occasions from CASADY2 where bulk tank milk was collected no more than 3 days before or after individual samples from all animals was performed. A total of 21 herds were represented in these sample occasions, 13 herds once, 6 herds twice and 2 herds three times. For each of

these sample occasions, the apparent prevalence of infected animals and the prevalence of faecal shedders were calculated in the whole herd and four age groups. The apparent prevalence of infected animals was calculated as the proportion of all animals in the herd or age group that were positive in the faecal culture test and/or the ELISA at cut-off 25 ODC%, which was the cut-off used to discriminate between positive and negative animals at both DVI and Steins Laboratory at that time.

Figure 4.13 (pp. 96-97) shows the relation between the apparent prevalence of infected animals and the bulk tank milk response against *Salmonella* Dublin in the whole herd and four age groups. It also shows the relation between faecal shedding and bulk tank milk response in the same groups. Because the data was limited for this part of the study, no statistical analysis was performed on this data, but factors accounting for most of the variation in bulk tank milk response related to the lactating cows were analysed in Manuscript 4. The findings illustrated in Figure 4.13 do, however, indicate that the apparent prevalence of infected animals among lactating cows explains the level of response in bulk tank milk to a much higher degree than the prevalence of infected animals among calves, young stock and heifers. In fact, it shows that it is possible to have a prevalence of up to 67% and a few faecal shedders among the calves when the bulk tank milk is low (below 25 ODC%). On the other hand, the bulk tank milk response was sometimes quite high, even though the apparent prevalence of infected animals among calves and young stock was low, and no faecal shedders were found. This situation may occur frequently in *Salmonella* Dublin infected herds, because the infection can be latent and only periodically cause clinical problems and shedding of bacteria.

Table 4.2 Serotypes of salmonella (*S.*) bacteria isolated in 35 herds and the classifications of these herds according to the Danish National Surveillance (DNS) program for *Salmonella* Dublin in May 2003. The registrations are from CASADY2,3 and 4.

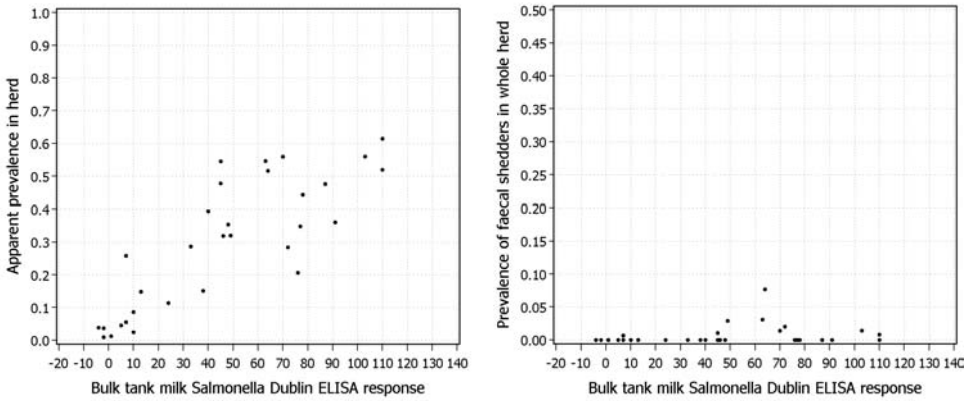
Herd no.*	Salmonella serotype(s) isolated	*Salmonella* Dublin level in the DNS-program***
1	*S.* Dublin, *S.* Derby , NTS**	2a
2	*S.* Dublin	2a
3	None	2b
4	*S.* Dublin	2a
5	*S.* Dublin	2a
6	*S.* Dublin	2a
7	*S.* Dublin, NTS	2a
8	*S.* Dublin	2a
9	*S.* Dublin	2a
10	None	2a
11	*S.* Dublin	2a
12	None	1
13	*S.* Dublin, NTS	2a
14	*S.* Typhimurium (DT170)	Stopped milk production in 2001
15	*S.* Dublin	2a
16	*S.* Dublin	2a
17	None	2b
18	*S.* Agona	1
19	*S.* Typhimurium (DTU312, DT193, DT120)	2a
20	*S.* Dublin	2a
21	*S.* Dublin	2a
22	None	Stopped milk production in 2001
23	None	2a
24	*S.* Dublin	2a
25	*S.* Typhimurium (DT 17)	1
26	None	Stopped milk production in 2002
27	None	1
28	None	1
29	None	1
30	*S.* Dublin	2a
31	None	2a
32	None	2a
33	*S.* Dublin	2a
34	NTS	2a
35	*S.* Typhimurium	2a

* The herd number refers to the herds described in Appendix B

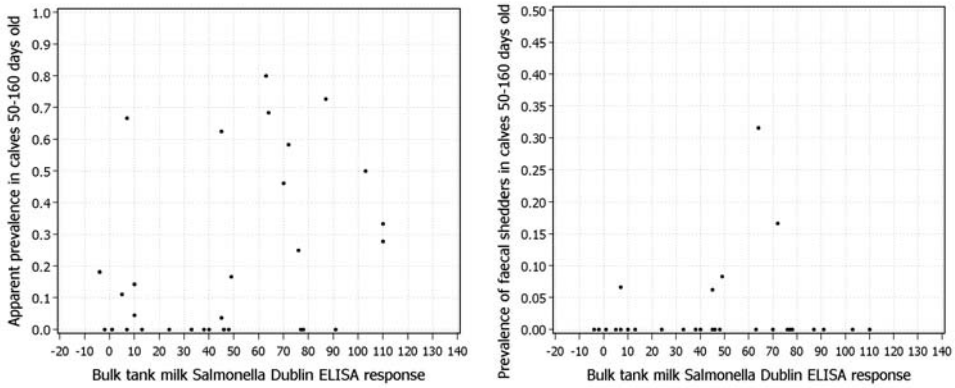
**NTS = non-typable salmonella

*** 1= Most likely free of *Salmonella* Dublin, 2a= Above cut-off value in bulk tank milk, 2b= unknown status due to too few samples provided for calculation of level

Whole herd



Calves (50-160 days of age)



Young stock (160-400 days of age)

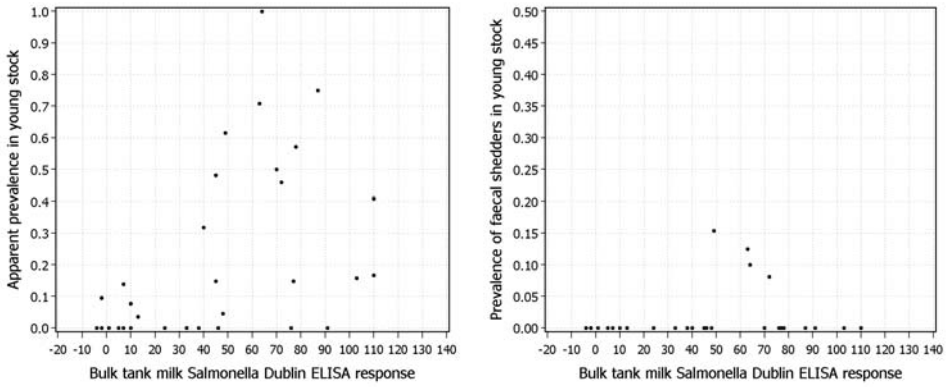


Figure 4.13 (full figure text on p. 97)

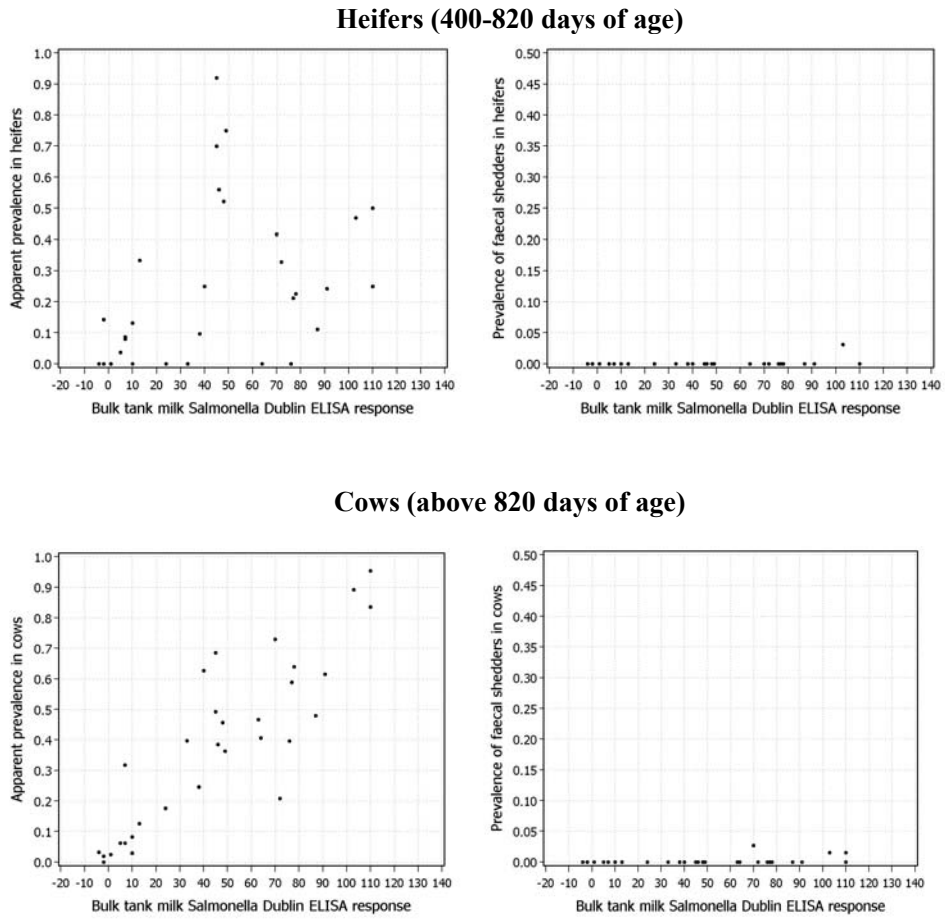


Figure 4.13 (pp. 96-97) Distribution of infected animals (measured as apparent prevalence according to individual ELISA response and prevalence of faecal shedders) in relation to bulk tank milk ELISA response against *Salmonella* Dublin illustrated for the whole herd and 4 age groups. The bulk tank milk samples were collected no more than 3 days before or after the individual samples.

Manuscript 3, ”What determines the variation in the bulk tank milk response against *Salmonella* Dublin in dairy herds?” is a study of factors describing the variation in bulk tank milk response of dairy herds. The study was based on repeated measurements from 30 herds. Three models were reported. These models all described the bulk tank milk ELISA response equally well and accounted for 77-78% of the variation in bulk tank milk ELISA response. The conclusions from that study were that factors influencing the outcome of the bulk tank milk ELISA response against

Salmonella Dublin were the antibody level of individual cows, the herd size, the number of cows with high individual ELISA response and the salmonella status of the herd.

The results from Manuscript 3 and the above considerations show that hypotheses 6 and 7 appeared to be true. The bulk tank milk *Salmonella* Dublin ELISA response mainly reflects the level of antibodies among the lactating cows, and to a smaller degree infection among calves and young stock. However, the results also showed that the bulk tank milk ELISA response is easier influenced by other factors in small herds than large herds and herds with few infected animals (low apparent prevalence). This is important knowledge for future interventions. Intervention strategies should comprise both culling of high responders and carriers, as well as general preventive measures to minimize transmission of the pathogen between animals and thereby lower the prevalence of infected animals over time. This task must be expected to be more time and work consuming in large and/or heavily infected herds. In known *Salmonella* Dublin infected herds, the bulk tank milk ELISA can be used as a guideline for how the intervention is proceeding. However, in herds where the true infection status is not known, the responses seen in the individual and bulk tank milk ELISA may come from infection with *Salmonella* Typhimurium as discussed in the beginning of this chapter (p. 67-68).

Chapter 5: Risk factors for *Salmonella* Dublin infection in dairy cattle

A risk factor is a host, agent or environment related factor that influences the probability that an event takes place. The event could be that an animal becomes infected and seroconverts or starts to shed bacteria, or that an animal becomes clinically ill from the infection. Usually, risk factors are determined using models including effects that determine an outcome. The outcome may be measured at different levels, i.e. animal or herd level. Also, the outcome variable may be measured at difference scales, i.e. continuous, ordinal or binary outcome. The difference in the type of outcome variable in risk factor models often makes it difficult to directly compare risk factor studies. However, together they may aid in building a picture of factors either preventing or increasing the risk of spreading the infectious organism between herds and animals, and they may aid in determining to which degree risk factors change the probability of infection. Host related risk factors could be age, sex, breed, the physiological state of the animals, which may again be related to for instance feeding strategies, vaccination status etc. Agent related risk factors might be for instance virulence, pathogenicity, infectiousness, antibiotic resistance and host specificity mostly determined by the genetic composition of the agent (the strain). Environment related risk factors are often related to management strategies in intensive production systems. For instance stocking density, type and amounts of feed, accessible water supplies, hygiene (infection load in the environment), usage of contaminated utensil, housing type, ventilation, flooded grassing areas, movement of animals, calving environment, in other words most things related to management and production facilities.

5.1 Introduction to risk factors for *Salmonella* Dublin

Risk factors for *Salmonella* Dublin infection can be evaluated at several levels. At herd level, the risk of introduction of the infection to a previously uninfected herd has been shown to be influenced by both management practices of the herd and location of the herd. Dutch specific pathogen free (SPF) dairy herds that were engaged in trade of living animals (or other types of direct contact between herds) and had a low level of biosecurity with regard to professional visitors to the herd, were shown to have increased risk of introduction of infectious organisms to the herd[117]. Overall, significant risk factors for introduction of important pathogenic infectious organisms (BVD, BHV1, Leptospira *hardjo* and *Salmonella* Dublin) into dairy herds were found to be (i) that cattle was allowed to return to the herd after unsuccessful sale (or other off farm activity) (OR=12.6), and (ii) that cattle was allowed to grass with cattle from other farms (OR=7.0). It was found to be a significant

protective factor if veterinarians always wore protective clothing from the farm when visiting the herd (OR=0.2). For *Salmonella* Dublin the most significant risk factor was found to be cattle grassing with cattle from other farms[117]. The weaknesses of that study were mainly a small sample size (a total of 95 herds of which 13 had one or more infections introduced during the study period) and a very low total incidence rate of 0.09 per herd year at risk. In a study of 1429 Danish dairy herds the results showed that the risk for a dairy herd to become infected with *Salmonella* Dublin (measured as a change from negative to positive bulk tank milk ELISA response) increased when nearby neighbours were also infected. Also the risk increased with the prevalence of seropositive herds in the geographic area[133]. Both studies were concerned with risk factors for a change in infection status from uninfected to infected with *Salmonella* Dublin based on serological diagnostic tests.

Risk factors at herd level may also be evaluated as factors influencing whether the infectious agent is present in the study population or not. In those risk factor studies there is usually no information about how long the infection has been present in the herds. One risk factor study in 100 feedlot cattle herds in the USA indicated that the practice of feeding tallow (a rendering by-product) and whole cottonseed or cottonseed hulls (all high in fat contents) within seven days prior to faecal sample collection was associated with an increased risk (OR=2.3 and 3.5 for tallow and cottonseeds, respectively) of finding salmonella bacteria in a pen[66]. A study of salmonella shedding in preweaned heifer calves in 1063 dairy herds in the USA revealed that medicated milk replacer (including one or more of the antibiotic compounds oxytetracycline, neomycin, chlortetracycline and decoquinate) and hay fed to dairy calves from 24 h of age until weaning, were associated with a reduced risk of salmonella shedding (OR=0.35 for medicated milk replacer and OR=0.52 for hay), as was calving in an individual area within a building (OR=0.53)[67]. None of the last two studies referred to here include information about what serotypes of salmonella bacteria were isolated from the herds. Therefore, the results may or may not apply to *Salmonella* Dublin. Both studies had a high risk of misclassification of negative herds due to the sampling procedures and methods used for detection of salmonella bacteria, and it is difficult to know the impact of such misclassification on the results.

A Dutch risk factor study for *Salmonella* Dublin infection on 126 dairy farms (29 case and 97 control farms) found the following risk factors for presence of *Salmonella* Dublin on the farm based on at least one culture positive result from the farm: Standardized herd size (OR=1.03), indicating that larger herds had a higher risk of *Salmonella* Dublin infection,

water surface in hectares (OR=2.06) indicating that where large areas of the farm pastures were surrounded by water streams (often used as drinking water), the risk of finding *Salmonella* Dublin on the farm was increased compared to herds with small or no water surrounding the pastures. This factor is mainly important for countries with cattle herds in wet lowlands with many natural streams, such as the northern part of the Netherlands, but may be relevant for cattle grassing very wet pastures anywhere in the world. Purchase of livestock from other herds was a risk factor (OR=4.29), as was liver fluke infection in the herd (OR=14.16) and solely grass feeding during the summer (OR=13.16). Contact to cows from other farms was found to be a protective factor (OR=0.07)[116]. This contact was defined as potential contact between cows over fences (Jan Veling, personal communication). The association between *Salmonella* Dublin and liver fluke infestation is supported by other studies, but the causal relationship is not entirely understood. It may be explained by liver fluke infestation increasing the susceptibility of cattle to *Salmonella* Dublin infection, thus prolonging the excretion of bacteria and increasing the tendency for carrier production with persistence in host tissues[4]. It may, however, also be because both *Salmonella* Dublin and *Fasciola* Hepatica multiply better under wet conditions and therefore share risk factors for presence in cattle herds.

At animal level risk factors can be evaluated as risk factors for becoming

- clinically ill from the infection (which requires clinical registrations)
- infected (which could be measured as faecal shedding or seroconversion)
- a carrier of the infection (which requires a definition for laboratory results from carriers and access to the required sampling and laboratory procedures)

Age is a known risk factor for becoming clinically ill from *Salmonella* Dublin infection[94,101]. Calves up to 12 weeks of age are unable to produce a strong and fast immune response to infection[21]. They are also referred to as immunonaive calves that are more susceptible to infections[10]. Very young calves (below the age of approximately 14 days) may be somewhat protected by passively transferred maternal antibodies from colostrum, but calves between 14 days and 3 months are more susceptible to the infection and therefore symptoms are most frequently seen in this age group of animals. This does not necessarily mean that this is the age group with the highest risk of becoming infected. In a herd endemically infected with *Salmonella* Dublin the bacteria can be found everywhere on the premises. It has been shown to be able to survive up to 6 years in dried faeces meaning that all animals in the herd are likely to be exposed to the bacteria[90].

Other important host related risk factors for clinical disease and for becoming a carrier animal are the conditions leading to stress and immunosuppression of the infected animal (transportation, parturition, BVD infection, compromised liver function e.g. with liver fluke infestation, deprivation of food, medical treatment etc.)[4,73,87,94,107,126].

Environment related risk factors for *Salmonella* Dublin infection directly related to the risk for the individual animal include housing system (loose housing increase the spread of the infection and expose each animal to more infectious organisms), stocking density and infection pressure of the immediate environment[94]. Therefore, the general hygiene in the barn section is important, but difficult to measure. Pastures recently contaminated with infected slurry is also an important risk for infection of individual animals[112,113].

Three risk factor studies are presented in section 5.2, 5.3 and 5.4. Study 3 in section 5.2 was initiated as a risk factor study of salmonellosis (defined as clinical disease most likely caused by *Salmonella* Dublin) in herds with outbreaks of *Salmonella* Dublin. Unfortunately, it turned out that clinical registrations and collection of blood and milk samples from 6 of the herds described in Chapter 3, pp. 58-61, in close relation to the estimated outbreak time were insufficient for the analyses to be completed. Therefore the analyses in Studies 3 and 4 were only performed for herd no. 30 for which thorough clinical registrations were available from the outbreak period. Study 4 in section 5.3 and Manuscript 4 (summary in section 5.4) are studies of risk factors for becoming a carrier under different circumstances.

5.2 Risk factors for salmonellosis in cows during an outbreak of *Salmonella* Dublin (Study 3)

Herd no. 30 experienced a serious outbreak of *Salmonella* Dublin starting in the cow barn in mid September 2001. The outbreak lasted approximately 5 months with highest incidence risk of salmonellosis (clinical disease with typical symptoms of *Salmonella* Dublin) in October 2001 (see Table 5.1) and the last cases among the adult cows in early January 2002. The cause of the outbreak was unknown. No purchase of animals had occurred for a two-year period before the outbreak. The herd did, however, have an outbreak of *Salmonella* Dublin among young calves approximately 6 years earlier, but the owners were apparently able to eliminate the infection fairly quickly by isolating all newborn calves on another farm and using strict hygiene measures to avoid the spread of the bacteria in the herd. It is questionable, if the infection was able to survive in latent carriers or in the environment after that

outbreak, and cause the new outbreak in 2001. Alternatively, the infection was introduced from outside the farm. Figure 5.1 shows the bulk tank milk ELISA response from the herd before, during and after the outbreak. At the time of the outbreak 138 animals were either lactating or dry cows/heifers that calved during the outbreak period. The owner recorded rectal temperature twice daily in October and November and clinical symptoms from all cows daily from September 2001 to January 2002 in order to catch early symptoms so that treatment could be initiated quickly. A total of 19 cows became ill with clinical symptoms that were typical of salmonellosis. Three of these cows aborted in the last trimester. Five other cows died from the infection after peracute or acute disease despite intensive treatment with fluids and antibiotics. *Salmonella* Dublin was diagnosed in early October 2001 from a cow with retained placenta from which material was submitted for bacteriological culture. A total of 21 faecal samples were culture positive when all animals in the herd were sampled in December 2001. These positive cultures came from all ages of animals, and they were not all from clinically ill animals. Clinical symptoms in the calves were not seen until the Summer 2002 when problems with neonatal diarrhoea occurred. The herd had three separated barn sections for adult cows, calves and young stock.

Table 5.1 Incidence and incidence risk for each month of the *Salmonella* Dublin outbreak in herd no. 30.

Month	Incidence	# cows at risk	Incidence risk
September	2	138	0.014
October	10	136	0.074
November	5	126	0.040
December	1	121	0.008
January	1	120	0.008

Hypothesis 8:

The risk of salmonellosis in cows during an outbreak of Salmonella Dublin is influenced by the lactation stage, parity and level of milk production at time of exposure.

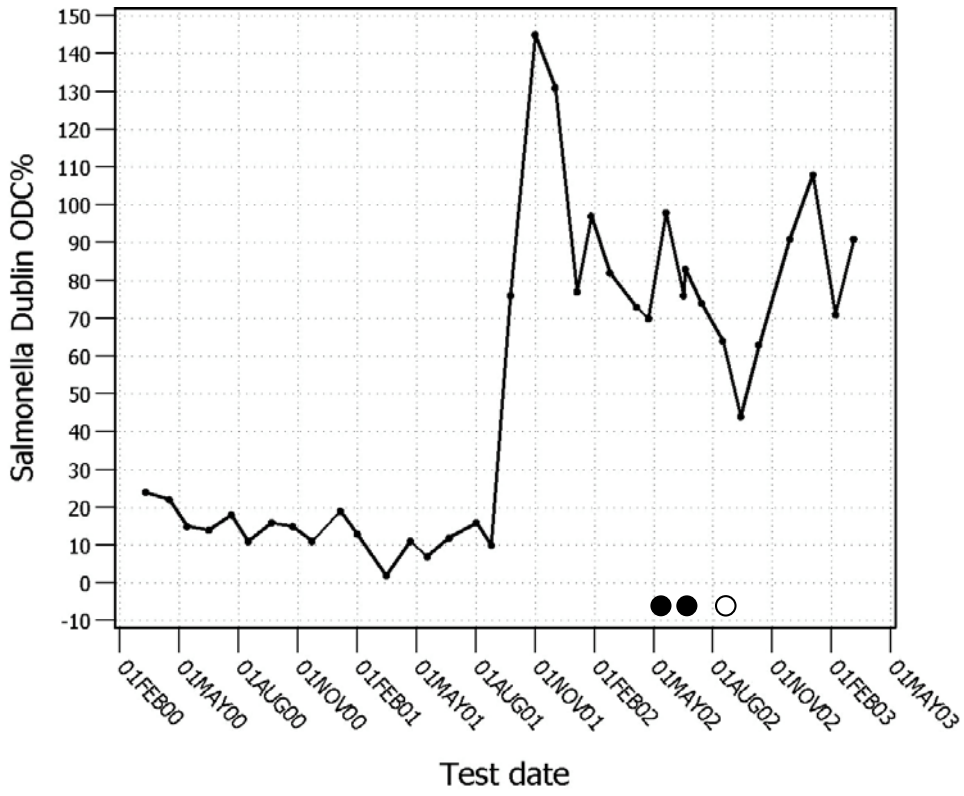


Figure 5.1 Bulk tank milk ELISA response in herd no. 30 before, during and after a severe outbreak of *Salmonella* Dublin starting in the cow barn in mid September 2001. ● indicate bacteriological culture positive dung pit samples, ○ indicate bacteriological culture negative dung pit sample.

The study was performed as a case-control study. Cases were all cows with one or more clinical registrations assumed to be caused by *Salmonella* Dublin (abortion, retained placenta when occurring together with other symptoms, diarrhoea and treatment for disorders of the digestive system, fever or death (not slaughter)). Controls were all animals that did not have any symptoms or that had only symptoms not assumed related to *Salmonella* Dublin (e.g. mastitis and lameness). Disease dates for cases were the first date for registered symptoms, for controls a pseudo date set to the 18th of October 2001, which was assumed to be the date with the highest risk of becoming infected.

The risk factor "lactation stage" was defined as a categorical variable with the following levels:

1) Animals 0-14 days from calving on the registered disease date
2) Animals 15-90 days from calving on the registered disease date
3) Animals 91-500 days from calving or dry cows/heifers on the registered disease date

The risk factor "parity" was directly extracted from CASADY5 and grouped into three levels:

1) Heifers and 1st lactation cows
2) 2nd lactation cows
3) Cows in 3rd or higher lactation

The risk factor "level of milk yield" was calculated from register data of CASADY5 as the mean energy corrected milk yield (ECM) registered from day 30 to 120 of the lactation before the disease date or pseudo disease date. ECM was calculated using the measured protein and fat contents of the milk and the amount of milk produced, by the following formula slightly modified from Sjaunja et al. (1990):

$$ECM = (\text{kg milk}) * (383 * \text{fat percent} + 242 * \text{protein percent} + 783.2) / 3140$$

This risk factor was used in the statistical analysis as a continuous variable. On a total of 128 out of the 138 cows, milk yield measurements were performed.

The statistical method was a multivariable logistic regression performed using the PROC GENMOD procedure in SAS® version 8.2. The outcome was salmonellosis during the outbreak period (yes/no). The initial model was the full model with all two-way interactions. Backward elimination was performed to reduce the model while checking each variable for confounding. Variables were considered confounders if the parameter estimates of other variables changed more than 25% by removal or reintroduction of the variable in question in the model. Table 5.2 shows the distribution of animals in the different categorical risk factors groups and Table 5.3 gives an overview of the milk yield in each of the categories. Table 5.4 and 5.5 show the results of the final model.

Table 5.2 Distribution of cows in the different categories of parity and lactation stage.

Variable	Category	Cows (N)	Cases (n)	Controls (n)	Incidence risk
All		138	19	119	0.14
Parity	1	43	5	38	0.12
	2	31	6	25	0.19
	>2	64	8	56	0.13
Lactation stage	0-14	15	11	4	0.73
(days post calving)	15-90	30	6	24	0.20
	91-500	93	2	91	0.02

Table 5.3 The distribution of the mean energy corrected milk yield (ECM) in the different categories of cows in herd no. 30 before the outbreak period

Variable	Category	Total number of cows	Mean ECM	
			Cases (Std*) (n=15)	Controls (Std) (n=113)
All		128	29.4 (5.1)	31.2 (6.2)
Parity	1	33	25.4 (N/A**)	27.4 (3.8)
	2	31	27.8 (2.4)	28.5 (5.6)
	>2	64	31.0 (6.3)	34.5 (5.7)
Lactation stage	0-14	10	28.7 (2.9)	24.3 (7.1)
(days post calving)	15-90	25	28.3 (3.3)	31.2 (6.8)
	91-500	93	35.0 (13.5)	31.4 (6.0)

*Std=Standard deviation of mean ECM

** Std cannot be calculated because n=1

The conclusions were that stage of lactation was a highly significant risk factor, whereas parity and level of milk yield did not appear to influence the risk of salmonellosis in cows during an outbreak of *Salmonella* Dublin in the study herd. Fresh cows (0-14 days post calving) had significantly higher odds of salmonellosis than cows in mid lactation (15-90 days post calving) and late lactation (>90 days from calving). Cows in mid lactation also had significantly higher odds of salmonellosis than late lactation cows. The findings may be explained by immunosuppression in fresh cows and negative energy balance of cows in early and mid lactation as described in other studies[15,59,73]. Also, in this particular herd, the calving pens were used as isolation pens for clinically ill animals during the outbreak period. This most likely caused a high infection pressure for the calving cows that entered these pens consecutively. This may also be part of the reason for the very high odds ratios found between lactation stages 1 and 3. Only two late lactation cows became clinically ill during the outbreak, which introduces high uncertainty in the study. The study only included one herd with an outbreak of *Salmonella* Dublin starting in the cow barn, so the conclusions may not apply to other herds experiencing an outbreak with a different infection spread pattern. Studies like this require detailed, reliable daily clinical registrations during the entire outbreak period, which may last ½-1 year. This is the major challenge in obtaining data for such studies.

Table 5.4 Results of the final model of risk factors for salmonellosis during an outbreak of *Salmonella* Dublin in a dairy herd

	Category	Estimate	S.E.	χ^2	p	P{salmonellosis}[a]	95% CI of P{salmonellosis}
Intercept		-3.818	0.715	28.52	<0.001		
Lactation				43.87	<0.001		
stage	0-14	4.829	0.923			0.73	[0.65 ; 0.81]
(days post	15-90	2.431	0.848			0.20	[0.13 ; 0.27]
calving)	91-500	0.000	0.000			0.02	[0.00 ; 0.04]

[a] Estimated probability of salmonellosis

Table 5.5 Odds ratios (OR) for salmonellosis between lactation stages during an outbreak of *Salmonella* Dublin in a dairy herd

Lactation stage		OR	95% CI for OR	p
0-14	15-90	11.0	[2.6 ; 47.0]	0.001
0-14	91-500	125.1	[20.5 ; 763.9]	<0.001
15-90	91-500	11.4	[2.2 ; 60.0]	0.004

5.3 Risk factors for becoming a carrier cow after an acute outbreak of *Salmonella* Dublin (Study 4)

A question often raised about *Salmonella* Dublin infection is: Why do some animals become carriers upon infection when others do not? The purpose of Study 4 was to examine risk factors for becoming a carrier upon infection during an outbreak.

Hypothesis 9:

> *The risk of becoming a carrier after an outbreak of Salmonella Dublin in a dairy herd is influenced by host and management related factors at time of infection*

Again, herd no. 30 was used because suitable data were available for the purpose. Only cows and heifers that calved during the outbreak period were included in the study. Cows were classified as carriers or transiently infected, based on *Salmonella* Dublin ELISA response in repeated milk samples according to recommendations from other studies[104,108]. The samples were collected from October 2001 to September 2002. In order to enter the study the cows had to have at least 4 samples over a period of at least 180 days. Carriers were defined as cows with an ELISA response above 60 ODC% in at least 4 samples (i.e. over a period of more than 92 days) and with the last sample being above 60 ODC%.

Transiently infected cows were defined as cows with an ELISA response that initially increased to above twice the mean baseline level of all animals before the outbreak (indicating infection and seroconversion in that animal) and then dropped to below 40 ODC% and was below this cut-off at the last sample. Examples of the two groups of animals are shown in Figures 5.2 and 5.3. A total of 39 cows were classified carriers and 57 were classified transiently infected. The rest of the 138 cows from the herd were either dead from

salmonellosis, slaughtered or did not have enough samples collected to be classified according to the criteria.

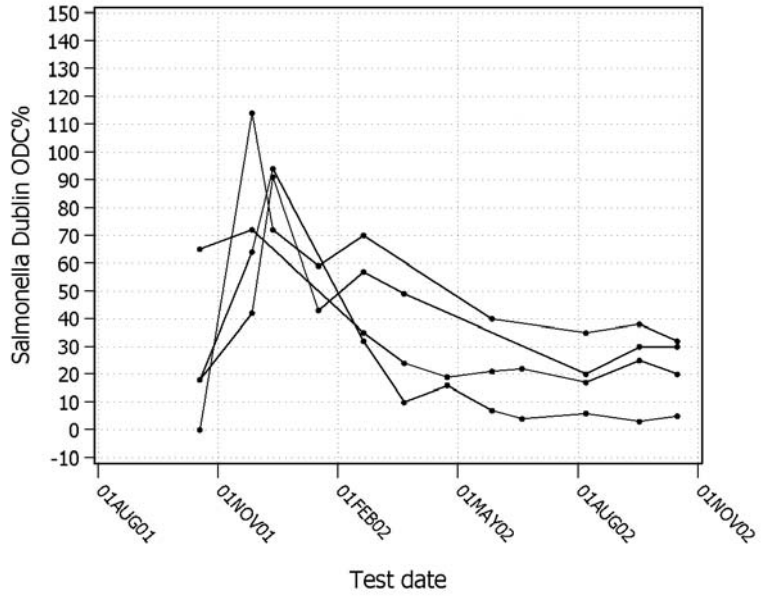


Figure 5.2 Examples of cows classified as transiently infected in herd no. 30 after an outbreak of *Salmonella* Dublin.

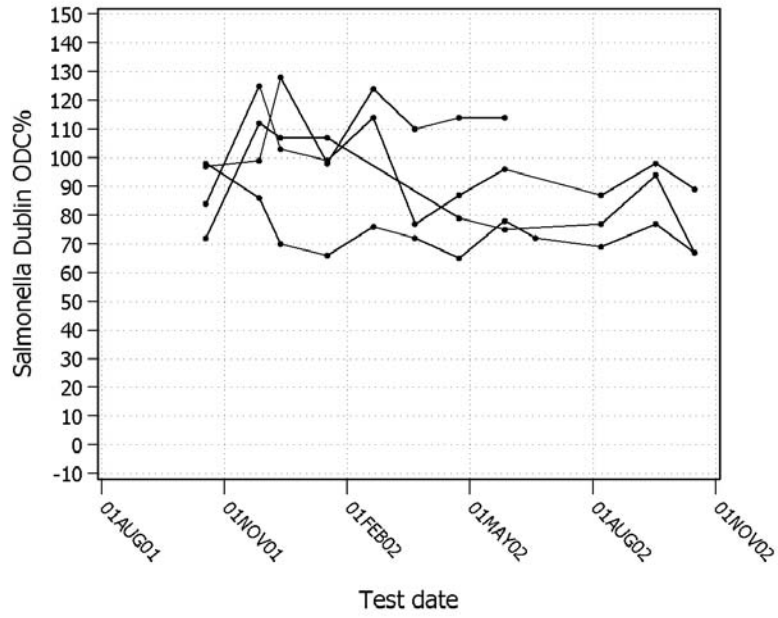


Figure 5.3 Examples of cows classified as carriers in herd no. 30 after an outbreak of *Salmonella* Dublin.

The study was a case-control study where risk factors were analysed using a multivariable logistic regression model. The following risk factors were included in the initial model:

- Time of calving in relation to the start of the outbreak:
  Group 1: Calved during the outbreak period
  Group 2: Calved 0-5 months before the start of the outbreak
  Group 3: Calved more then 5 months before the start of the outbreak
- Parity:
  Group 1: Heifers and 1st lactation cows
  Group 2: 2nd lactation cows
  Group 3: Cows in 3rd or higher lactation

- Mean energy corrected milk yield before the disease date or pseudo disease date was calculated as described in Study 3.

- Salmonellosis (yes/no): Indicates if the cow was ill from salmonella during the outbreak.

- Antibiotic treatment (yes/no): Indicates if animals were treated with systemic procainpenicillin or oxytetracycline from one week before to one month after the disease date (for cases) or pseudo disease date (for controls).

The statistical method was performed using the PROC GENMOD procedure in SAS® version 8.2. The initial model was the full model with all possible two-way interactions. Backward elimination was performed to reduce the model while checking for confounding factors. Interactions with salmonellosis and antibiotic treatment were not possible to estimate because of the low number of diseased and treated cows. Tables 5.5 and 5.6 show the distribution of carriers and transiently infected in relation to the variables for analysis. Table 5.7 shows the results from the final model. It was not possible to analyse both antibiotic treatment and salmonellosis the same model due to confounding between the two variables.

Table 5.5 Distribution of carriers (cases) and transiently infected cows (controls) in the different groups of the risk factors included in the start model.

Variable	Category	Cows	Number of cows		Incidence risk of C
			C[a]	T[b]	
All		96	39	57	0.41
Parity	1	41	18	23	0.44
	2	17	8	9	0.47
	>2	38	13	25	0.34
Calving time	During outbreak	36	18	18	0.50
	0-5 months < outbreak	41	15	26	0.37
	>5 months < outbreak	19	6	13	0.32
Salmonellosis	Yes	9	7	2	0.78
	No	87	32	55	0.37
Antibiotic treatment	Yes	17	8	9	0.47
	No	79	31	48	0.39

[a] Carrier cows
[b] Transiently infected cows

Table 5.6 Mean energy corrected milk yield (ECM) of carriers (cases) and transiently infected cows (controls).

Variable	Cows	Mean ECM	S.E.	Mean ECM	
				C (n=28)	T (n=50)
Mean daily milk yield (ECM)	78	31.0	6.23	31.0	30.9

Table 5.7 Results from the final model of risk factors for becoming a carrier during an outbreak of *Salmonella* Dublin in herd no. 30.

Variable	Category	Estimate	S.E.	χ^2	p	OR	95 % CI of OR
Intercept		-0.542	0.223	5.93	0.02		
Salmonellosis				5.70	0.02		
	Yes	1.794	0.832			6.0	[1.2 ; 30.7]
	No	0.000	0.000			1	-

The conclusions were that salmonellosis during a *Salmonella* Dublin outbreak is an important risk factor for becoming a carrier of the bacteria. Antibiotic treatment is also a possible a risk factor, but salmonellosis was a confounder of the antibiotic treatment effect in this study and both variables could not be estimated in the model at the same time. However, in the model containing antibiotic treatment as the only explanatory variable for becoming a carrier, the effect of this variable was not statistically significant (χ^2=0.35, p=0.553) with this data. Parity, time of calving in relation to the outbreak and mean energy corrected milk yield before the outbreak did not appear to be risk factors for becoming a carrier in this study. The findings are supported by another study which found that salmonellosis in very young calves lead to a persistent carrier state where bacteria were located in lymph nodes up to one year after the period with clinical disease[53].

5.4 Risk factors for becoming a carrier in endemically infected herds

A risk factor study was performed for 12 dairy herds using data from CASADY2, CASADY3 and CASADY4 to determine risk factors for becoming a carrier in endemically infected herds (Manuscript 4). Herd no. 30 was included in the study, because it was considered endemically infected after the initial outbreak period of 5 months. The herds were selected from the 35 herds by the following criteria: *Salmonella* Dublin had to be isolated from at least one faecal sample during the study period. Also, every herd had to have a minimum of 2 animals in each of the outcome groups, carriers and transiently infected. Animals allowed in the study had to have at least 4 samples available over a period of 270 days, and the first sample event had to be negative so that an infection date could be estimated. For this study carriers were defined as animals with positive test results (serum ELISA > 35 ODC% and/or milk ELISA > 48 ODC% and/or seroconversion since last

sample and/or positive faecal culture) for a minimum of 240 days with the last sample being positive. Transiently infected animals were defined as animals with positive test results for a maximum of 90 days and where the last sample was negative. A total of 157 animals were classified as carriers and 87 were classified as transiently infected.

Hypothesis 10:

> *Reduced immune capacity of young calves and animals under increased stress load such as around the time of calving may increase the risk of animals becoming carriers instead of recovering, when they are infected with Salmonella Dublin.*

The statistical analysis was performed using a multilevel, multivariable logistic regression model using backwards elimination from a full model with 2-way interactions. Herd was included as a random effect in the model. The software used was the GLIMMIX macro in SAS® version 8.2.

The variables tested in the model were:

- Herd as random effect
- Age and time from calving, which was a categorical variable with the following groups:
    1) Calves below one year of age
    2) Heifers above one year and up to 70 days before 1st calving
    3) 1st parity cows and heifers in the period 1st calving ± 70 days
    4) 1st parity cows after 70 days into 1st lactation and up to 70 days before 2nd calving
    5) 2nd or higher parity cows in the period calving ± 70 days
    6) 2nd or higher parity cows after 70 days into lactation and up to 70 days before next calving
- Season, which was divided into quarters of the year
- Age group prevalence (continuous variable)
- Herd prevalence (continuous variable)

The resulting model included herd, age and time from calving, season and herd prevalence without any interactions (Table 3 in Manuscript 4). The results showed that animals that

become infected as heifers (between one year of age and up to first calving) or as cows close to time of calving are more likely to become carriers than calves and cows in mid or late lactation. The results also indicated that the lower the herd prevalence of potential shedders, the more likely animals are to become carriers upon infection. Also, season may influence the risk of becoming a carrier upon infection with the highest risk being in late winter and spring. This factor was, however, borderline significant and since only two years were represented it is difficult to generalize the findings. The herd effect indicates that animals in some herds have an overall higher risk of becoming carriers than animals from other herds (Figure 5 in Manuscript 4). The results are discussed in Chapter 6 together with results from the four studies and the other three manuscripts.

Chapter 6: General discussion, conclusions and perspectives

6.1 Introduction

This thesis is about diagnostic tests and risk factors related to *Salmonella* Dublin infection in dairy cattle. The focus has been on developing a better understanding of how to interpret results from commonly used diagnostic tests, such as ELISA used on individual serum and milk samples and ELISA for bulk tank milk samples. Also the use of conventional bacteriological culture of faecal samples has been evaluated. These tests have been used to gain new information about risk factors for becoming clinically ill from *Salmonella* Dublin during outbreaks and for becoming a carrier animal upon infection with *Salmonella* Dublin in both outbreak herds and endemically infected herds.

6.2 Data quality

The epidemiological investigations and test evaluations performed in this thesis were based on data and laboratory results collected through the "Integrated Cattle Health and Milk Quality Project", which was limited to participation of 110 dairy herds in the so-called Kongeå-region in the Southern part of Jutland, Denmark[5]. Of these 110 herds, 35 were used in the present thesis. It may be criticised that this sample of dairy herds is not representative for the rest of the Danish cattle population, and that the sample of herds is rather small. However, the 35 herds represent herds of different sizes, barn and management types, breeds and most importantly herds with the full range of *Salmonella* Dublin infection levels – measured as prevalence of serologically positive animals and number of shedding animals. Also, the true infection status of most of the herds was not known at time of selection for the project. In the sample of herds there is an overrepresentation of salmonella positive herds compared to the rest of the country, where the prevalence of infected herds is currently estimated to be around 23% according to the Danish National Surveillance Program for *Salmonella* Dublin. Therefore, the sample of herds should not be used to determine herd sensitivity and specificity of diagnostic tests at herd level, and it is not suitable for risk factors studies at herd level. Such studies have been performed by others, especially in the Netherlands, and there is no reason to believe that the conclusions made in those studies are not applicable to Danish conditions[116,119,120].

The focus of the thesis was related to the individual animals and transmission of the pathogen between animals (i.e. within the herd). Due to the complex pathogenesis of *Salmonella*

Dublin and the lack of long-term immunity to the infection, animals may switch back and forth between different stages of infection (e.g. uninfected, actively shedding, latent carrier). Therefore, estimation of the infection status of an animal requires repeated sampling of the animal over time. To relate the infection status of an animal to the population of cattle around it, sampling of all animals in the age group or herd is required. This type of sampling is very costly and time-consuming, and also requires dedicated farmers that will allow intensive sampling in their herds. Therefore, this type of intensive sampling is rarely performed as field studies in a large number of herds. Seen in this perspective, the sample size in the present study is fairly large and provides detailed information from the herds that makes it possible to gain new knowledge about infection stages, risk factors and dynamics of the infection within the herds. Much time and effort has been used on data error corrections and data editing to provide a reliable database "CASADY" for the studies performed in this thesis as well as for future studies. The datasets of "CASADY" are suitable for evaluation of test performance of ELISAs and bacteriological culture methods in the field.

6.3 Interpretation of diagnostic test performance

In this thesis, ELISAs for use on milk and serum samples from cattle were evaluated with regard to sensitivity and specificity for detection of *Salmonella* Dublin infected animals using two different methods, a classic validation method using faecal shedding as a gold standard reference test and a latent class method avoiding the use of gold standard reference tests. The latent class method also provided a rough estimate of the sensitivity of the faecal culture test. Further, it was evaluated what effect pooling of faecal samples have on the faecal culture test validity. The overall conclusions are:

- The diagnostic sensitivity of the bacteriological faecal culture test used in this thesis is poor. The sensitivity is decreased further by use of pools from five animals for detection of *Salmonella* Dublin infected animals in dairy herds. Rough estimates of the sensitivity are in the range 6-14% for the pool-first test method.

- The serum ELISA validity is significantly better when used for detection of *Salmonella* Dublin infected animals in the group of calves and young stock aged 100-299 days than when used for younger calves or older animals. The test sensitivity (Se) is very poor for calves under the age of 100 days when using 25 ODC% or 50 ODC% as the cut-off value (Se=46% and 21%, respectively).

- Serum and milk ELISAs perform almost equally in adult cows. However, the specificity at the cut-off values used by laboratories in Denmark today are overestimated by the classic test validation method, where samples used as gold standard negative references solely consist of samples from known uninfected herds. Likewise, the sensitivity may be underestimated by the classic test validation method when only bacterial faecal shedders are included as gold standard positive references.

Using the two-way sensitivity and specificity plots provided in this thesis it is possible to read the validity estimates that can be obtained at different cut-off values. This can be used to optimise the cut-off values to the purpose of testing. If, for instance, the purpose of testing is to obtain an estimate of prevalence of infected animals in a herd and the cut-off value used is 25 ODC% for all animals, a true prevalence estimate can be obtained for each age group by adjusting the apparent prevalence of infected animals by the sensitivity and specificity estimates at a cut-off of 25 ODC%. If, however, the purpose of testing is to detect persistently infected animals (carriers) in order to cull these, the cut-off value may be set higher to avoid too many false positive results, for instance at 50 to 60 ODC%. Animals should be tested more than once with a reasonable interval in order to detect those animals that are most likely persistently infected and not just recovering from infection. It should be noted that carriers are easier to detect by the use of ELISA than are acutely infected animals[121] (i.e. the sensitivity of the ELISA is much higher for carriers than acutely infected animals). Other studies have suggested optimal test scenarios for detection of persistent carriers[104,108]. However, the titre values used in those studies are not directly transferable to the ODC%-values used at the laboratories in Denmark today. Therefore, the optimal ODC% cut-off value to use for detection of persistent carriers still remains to be determined, and it may in fact be a matter of choice of the person requesting the laboratory result. Therefore, the ODC% value is advantageous as a laboratory result as opposed to a qualitative infected/non-infected type of laboratory test results from the ELISAs. In herds with a high prevalence of infected animals, the farmers and advisors could decide to use a high cut-off value in the beginning of an intervention plan, and to cull those animals that remain above this cut-off value for more than 4 months in compliance with the recommendations by Smith et al. (1993). This minimises the costs and problems with finding replacements for the culled animals. At the same time management strategies to reduce transmission of *Salmonella* Dublin between animals and to reduce the contamination of the barn environment should be initiated. The models from Manuscript 3 illustrate that ignoring such

management strategies in infected herds are likely to lead to unsuccessful intervention. After the prevalence of infected animals is reduced, the intervention strategy can be re-evaluated and the cut-off value can be lowered to optimise the predictive values.

A question often raised, after the National Surveillance Program for *Salmonella* Dublin was initiated, is whether it is possible to certify individual animals free of *Salmonella* Dublin based on ELISA tests. As illustrated in Figure 1 in Manuscript 2, the negative predictive value of the serum ELISA is estimated to be between 88% and 97% at cut-off 25 ODC% for animals aged 100 days or more in herds with less than 20% prevalence. At higher herd prevalence the negative predictive value decreases. As illustrated in Manuscript 3, dairy herds with bulk tank milk ELISA responses above 25 ODC% may very well have prevalences of infected cows higher than 20%. In other words, the negative predictive value is not perfect - even at low herd prevalence - and the test cannot be used to certify an animal free of *Salmonella* Dublin. This issue could be dealt with by (i) lowering the cut-off value even further and thereby increasing the negative predictive value, (ii) combining the bulk tank milk ELISA response with individual animal testing or (iii) only certifying animals in quarantine that are tested twice with 2-3 week intervals, and thereby allowing the ELISA response to raise if infection was in fact present in the tested animal. However, due to the suboptimal negative predictive value it may only be possible to certify animals as "low risk" animals instead of "free of *Salmonella* Dublin". It is a question of political decisions that could be discussed in the cattle industry, whether these are options to be introduced into the surveillance system in order to allow more freedom of movement of low risk animals without consequences for the classification level.

It is very likely that a number of Danish dairy herds are currently being classified incorrectly as *Salmonella* Dublin level 2a, because the specificity is not perfect. Assuming that the specificity estimate of 98% provided by Veling et al. (2001)[120] is similar to the specificity of the bulk tank milk ELISA used in the Danish National Surveillance Program, approximately 30 Danish dairy herds are currently being falsely classified as most likely infected with *Salmonella* Dublin. If the proportion of *Salmonella* Typhimurium infected herds is higher in Denmark than in the Netherlands, the number of falsely classified herds may be substantially higher. The fact that level-classification is based on four bulk tank milk samples collected over a period of one year increases the sensitivity, but also decreases the specificity. This would lead to a larger number of herds falsely classified as *Salmonella* Dublin level 2a.

6.4 Risk factors and carriers of *Salmonella* Dublin

Salmonella Dublin can be considered a multifactorial disease. It can be present in a herd without any obvious clinical symptoms, and it can cause a wide range of severity of disease under the different circumstances. It may cause severe outbreaks when entering fully susceptible herds, it may become endemic with low incidence and occasional outbreaks of disease, and it is even possible that it disappears from a herd without any further intervention directed against it. When studying risk factors for multifactorial diseases, risk factors can be associated with three more or less overlapping parts of a triad: Risk factors related to (i) the pathogen, (ii) the host and (iii) the environment as illustrated in Figure 6.1.

The pathogen itself is a necessary cause for salmonellosis and for production of persistent carriers, but certain genetic traits in different strains may lead to differences in for instance virulence. Studies have suggested the importance of the cell-mediated immunity in clearing salmonella infections[19,97,114]. At the same time, it has been suggested that the cellular immunity is suppressed while the humoral immunity is activated during acute bovine salmonellosis[61]. Results from Chapter 5 showed that the risk of becoming clinically ill during an outbreak of *Salmonella* Dublin was significantly higher for cows in peripartum, and that the risk of becoming a persistent carrier of *Salmonella* Dublin was significantly higher in animals that had been clinically ill and in animals that had become infected close to calving or as heifers. Had it been possible to test direct measures of cellular immunity, this may have aided in understanding the findings in relation to the pathogenesis of *Salmonella* Dublin. The role of the cellular immunity and the pathogenesis of *Salmonella* Dublin is still being debated in literature.

Haptoglobin is an acute phase protein that has been shown to be useful as an objective marker of severity of salmonella infection in cattle[72], but its production may also be induced by other infections, trauma and by stress after e.g. transportation. Stress-induced haptoglobin production has been shown to be associated with a marked inhibition of lymphocyte function[77] and an increased susceptibility to salmonella infection. It may be useful to measure haptoglobin levels in cattle around time of infection with *Salmonella* Dublin to test the effect of high haptoglobin levels on the risk of becoming a carrier animal.

The studies in Chapter 5 and Manuscript 4 illustrated the importance of factors related to the time of infection with *Salmonella* Dublin. However, it was not examined whether factors related to a later stage of infection (i.e. 1-3 months after initial infection) was related to the

risk of becoming a carrier. It is not unlikely that factors such as disease, movement to new pens, antibiotic treatment, stress, negative energy balance and high milk production in the months after the initial infection are important determinants of whether the animals is able to clear the infection or whether they become persistent carriers.

Studies have suggested that natural resistance to intracellular pathogens such as *Salmonella* Dublin, *Mycobacterium bovis* and *Brucella abortus* was found to be heritable and passed on from parent to offspring. The bovine NRAMP1 gene was found to be one of the major candidate genes involved in the intracellular control by in vitro macrophage studies[1,2,28]. This is another factor influencing the course of infection in individual animals. In the present thesis it was not possible to include host genetics as part of the risk factor studies in Chapter 5. However, it is recommended for further studies.

Many of the environment related risk factors for *Salmonella* Dublin infections have been studied elsewhere[66,67,73,115-117,133]. In this thesis the prevalence of infected animals was found to be a protective factor for becoming a carrier. In other words, the highest risk that animals would become a carrier upon infection, was when they became infected under low prevalence conditions. There are several possible explanations for this phenomenon. First, under low prevalence conditions only few animals will become infected.

It is possible that those animals that do become infected under conditions with low infection load are those that are most susceptible to infection and therefore also more prone to becoming carriers. An alternative hypothesis is that when the infection dose is low the cellular immunity is not stimulated sufficiently to clear the infection, which in return may allow the organism to establish a latent infection in the host. Also, clearance of infection is a process going on for several months. Many animal and herd related events might occur during this period and possibly lead to a change in the course of infection in the individual animal. Very few studies are available to support or rule out these hypotheses. Therefore, more research is needed to illustrate the mechanisms behind the development of persistent carrier cattle.

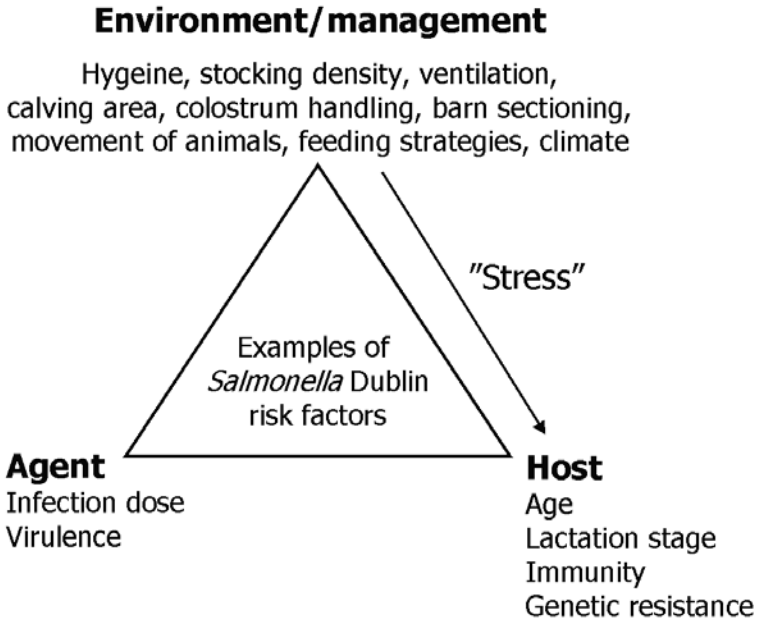


Figure 6.1 Examples of risk factors determining the outcome of infection with *Salmonella* Dublin in cattle.

6.5 Infection dynamics within dairy herds

Dynamics of infectious diseases are often described by mathematical models, which are useful for combining data and prior knowledge to achieve a better understanding of the dynamics of infectious diseases (e.g. Markov chain and Reed-Frost SIR-models)[37]. Such models are also useful for predictions and decision support. For instance, the effect of intervention on R_0, which is the so-called basic reproduction parameter indicating the number of new infected animals per infectious animal in barn sections or the whole herd, can be demonstrated by simulation trials. Infection dynamics models describe the relations between parameters (e.g. infection and recovery rate) and variables (e.g. the number of susceptible, infectious and immune animals in the study population) at each point in time with regard to transmission of a pathogen between animals. The parameters depend on the prepatent period (i.e. the period between infection of the host and the earliest time at which the causative agent can be recovered from the host[12], which then becomes infectious to other individuals), the virulence of the pathogen, available infection routes etc. The relations between the variables depend on the parameters and risk factors influencing the susceptibility of animals to infection, their ability to recover or become immune, birth rate, purchase rate, mortality and

culling rate. These parameters and risk factors need to be studied in more detail for *Salmonella* Dublin before mathematical models of within herd infection dynamics can be developed and tested.

Quantified host, pathogen and environmental risk factors for infection characteristics together with laboratory test results from all animals in the study population can be used to determine the size of these parameters and variables in such models. However, as is often the case for infectious diseases, information about infection rate parameters and risk factors are limited for *Salmonella* Dublin except for incubation period, prepatent period and susceptibility of different age groups. These are reasonably well described from experimental studies[43,96,101]. There are also a few known, but not quantified risk factors for infection such as season and climate[109,131] as well as concurrent disease[4]. The prevalence of animals testing serologically positive in different age groups of infected herds have been determined by cross-sectional studies[119] but may vary a lot between herds. In order to obtain the variables, risk factors and parameters needed to estimate R_0, frequent sampling of all animals in a large number of herds over extended periods of time is required. Such studies typically require a considerable amount of financial support and a high level of organisation and quality control. Ample laboratory facilities for performing large numbers of diagnostic tests reliably are also necessary. The Kongeå-project provided the optimal practically possible fulfilment of these requirements through a rarely found constellation of project participants from both milk producers and the dairy industry, practicing veterinarians, private and public laboratories, universities and research institutions.

In combination, the results of the present thesis and results from the literature can be used to determine probability distributions between different infection stages in a mathematical model of *Salmonella* Dublin infection dynamics within dairy herds. Figure 6.2 illustrates one suggestion for the structure of such a model. It is the intention of the author of this thesis to continue developing the model by continuously adding knowledge about *Salmonella* Dublin pathogenesis, transmission probabilities, risk factors etc. It is the intention that the model can eventually be used to simulate the effect of intervention strategies in individual dairy herds as part of intervention trials in *Salmonella* Dublin infected dairy herds. In Figure 6.2 different stages of infection are shown together with the laboratory results that suggest which stage of infection the animals most likely belong to. When collecting data for the model building process, the interval between sampling events should preferably be so short that changes from one infection stage to another can only occur once per animals

between sample events. In reality this is almost impossible to obtain, because it means that the whole herd should be sampled not less than twice a month (e.g. an animal can move from susceptible to infectious and to immune/recovering in 2 weeks). Alternatively, less frequent samples can be collected and the probabilities for moving between stages and the time period spent in each stage can be estimated from the data. Different options can be tried in the model to see which options fit the data best. The probability for each animal to move between infection stages will depend on host and environment related risk factors. The time spent in each stage can be estimated from both the data and laboratory results and from known risk factors and experimental studies. The data collected for "CASADY" provide a good basis for such model building. CASADY2 contains data for all animals over an extended period of time, and provides data for estimation of time spent in each infection stage.

CASADY3 contains data for calves and adult cows collected frequently enough to estimate in more detail the probability of transmission from dam to calf under different conditions, the probability of transmission between calves under different housing types and the probability of infection of adult cows after calving. However, CASADY does not provide frequently collected data from young stock and heifer close to calving. Finally, it does not provide data to suggest the prevalence of mammary gland carriers in Danish dairy herds or how to diagnose this group of animals. Therefore, more frequent sample collection from all animals in the herds, including aseptic collection of milk samples for bacteriological examination is required if the model is to be extended to include the whole herd.

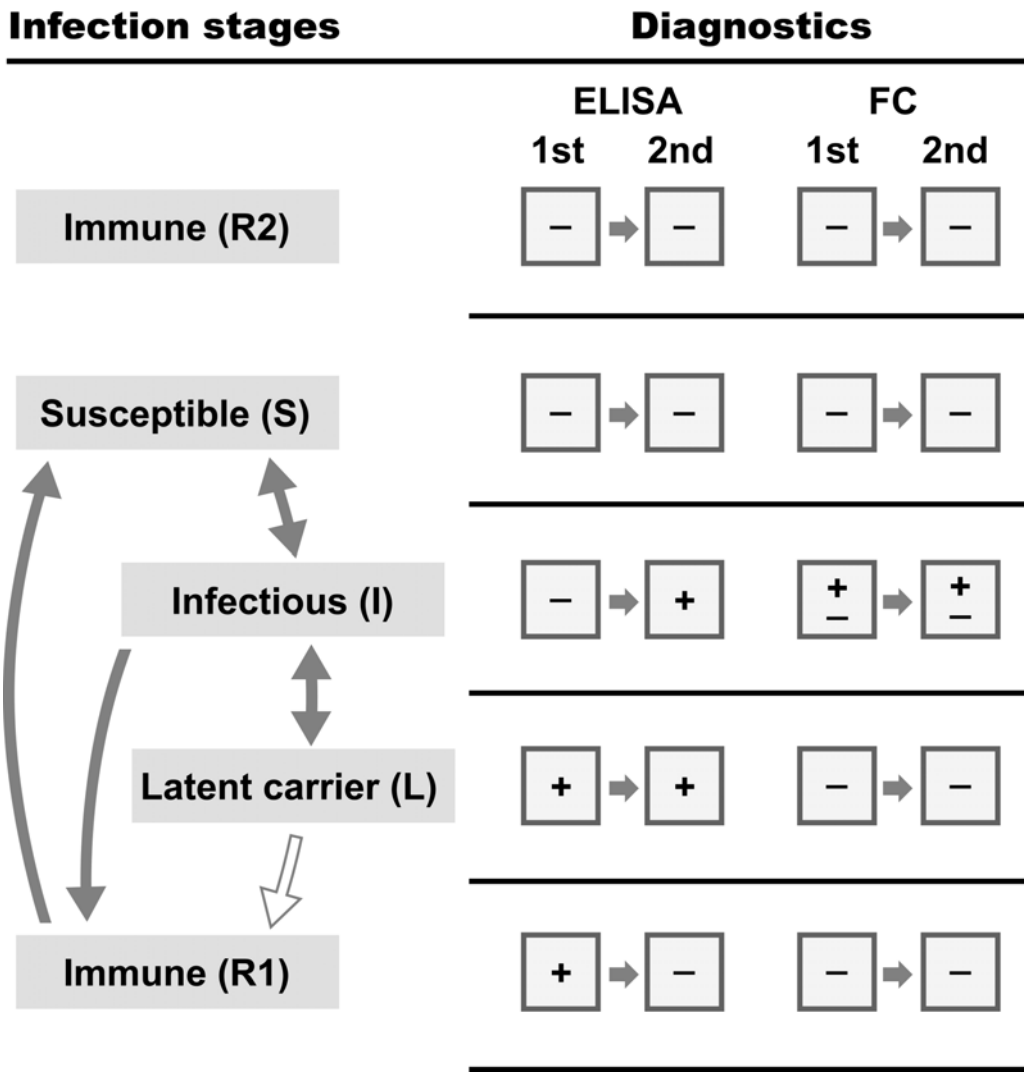


Figure 6.2 A schematic presentation of a theoretical infection dynamics model for *Salmonella* Dublin within dairy herds. To the left are different stages of infection. To the right, the change in diagnostic laboratory results between two sampling events that can be used to suggest which stage the animals most likely belong to. 'S' are animals fully or partly susceptible to infection. 'I' are animals infectious to other animals (acutely infected, reactivated or active carriers), 'L' are latent carriers that can move back and forth between being latent and infectious. It is unknown if latent carriers can move to the 'R1' stage. 'R1' are animals recovering from infection. 'R2' are animals genetically resistant to infection.

6.6 Recommendations

- Faecal culture should be used with caution for diagnosis of *Salmonella* Dublin. If a bacteriological diagnosis is required, targeted samples should be taken to increase the probability of finding an animal that is shedding (e.g. faecal samples from cows with very high antibody levels at time of calving, or faecal samples from acutely clinically ill, untreated animals). Large samples of more than 25 g should be obtained and the faecal material should be homogenised thoroughly before pre-enrichment. Probably collection of several samples during a day would increase the probability of detecting shedding of bacteria.

- It is strongly recommended to perform test optimisation and validation to increase the validity of the ELISA to avoid the current problems with cross-reactions and insufficient specificity in the ELISA. A better specificity of the currently used *Salmonella* Dublin ELISA for serum and milk may be obtained by optimising the antigen solution, so that the antigen is based solely on factor 9 of the O-antigen, or by performing follow-up ELISAs using factor 9 based O-antigen on samples with a positive test result in the original ELISA. It is also possible that using other conjugates based on a more narrow range of isotypes (e.g. IgG_2 or IgG_1) may improve the performance of the indirect *Salmonella* Dublin ELISA for serum and milk.

- The use of different cut-off values depending on age of the tested animals, purpose of testing and the herd prevalence would increase the validity and predictive values of the ELISAs and facilitate the use of these tests in epidemiological studies, intervention, certification and surveillance.

6.6 Suggestions for future research

- Studies of the effect of weight and homogeneity on the analytical sensitivity of the faecal culture test of *Salmonella* Dublin in faeces from calves and cows are needed. Also, it is relevant to study the effect of adding substances inhibiting the activity of the competing microflora from ruminants, so that the salmonella bacteria may grow better on the test media.

- Studies of the effect of intervention strategies including test-and-cull procedures as well as management strategies to reduce transmission between animals in cattle herds are required.

- More research is needed to understand the mechanisms determining which animals become persistent carriers of *Salmonella* Dublin and why. Inclusion of stress markers, such as haptoglobin, may aid in understanding the pathogenesis of the pathogen in relation to carriers. Also, studies of the host genetics behind resistance to the infection are needed.

- Studies of the reasons for differences in ELISA responses of serum and milk obtained on the same day from the same cow are needed. Indications from other studies suggest that mammary gland carriers may give rise to differences in local mammary and systemic antibody levels, but more work needs to be performed that includes bacteriological culturing of milk samples and preferably autopsy with subsequent organ cultures in order to evaluate the ELISAs in relation to different types of carriers.

- Studies of the prevalence and detection of mammary gland carriers in Danish dairy herds and the risk of transmission from this group of animals are required.

References

1. Adams, L.G., Barthel, R., Feng, J., Qureshi, T., Piedrahita, J. and Templeton, J.W., 1996. Genes associated with innate killing of Brucella abortus and Mycobacterium bovis by macrophages from genetically resistant cattle. Veterinary Immunology and Immunopathology 54, 135

2. Adams, L.G., Barthel, R., Gutierrez, J.A. and Templeton, J.W., 1999. Bovine natural resistance macrophage protein 1 (NRAMP1) gene. Archiv Fuer Tierzucht 42, 42-54.

3. Aitken, M.M., Hughes, D.L., Jones, P.W., Hall, G.A. and Collis, K.A., 1978. Effects of intravenous *Salmonella Dublin* on cattle at different stages of *Fasciola Hepatica* infection. Journal of Comparative Pathology 88, 433-442.

4. Aitken, M.M., Jones, P.W., Hall, G.A., Hughes, D.L. and Brown, G.T., 1981. Responses of fluke-infected and fluke-free cattle to experimental reinfection with Salmonella dublin. Research in Veterinary Science 31, 120-126.

5. Andersen, H.J., Aagaard, K., Skjøth, F., Rattenborg, E. and Enevoldsen, C., 2000. Integration of research, development, health promotion, and milk quality assurance in the Danish Dairy Industry. *In*: Salman, M.D., Morley, P.S., Ruch-Galie, R. (Eds.), Proceedings of the 9th Symposium of the International Society of Veterinary Epidemiology and Economics., pp. 258-260.

6. Anderson, M. and Blanchard, P., 1989. The clinical syndromes caused by Salmonella infection. Veterinary Medicine 84, 816-819.

7. Anon., 2002. Anvisning i fastsættelse af Salmonella Dublin niveau (CIR nr 9451 af 04/10/2002, Forskrift vedr. BEK Nr. 801 af 26/09/2002). Fødevaredirektoratet Danmark, 1-12.

8. Anon., 2002. Bekendtgørelse om *Salmonella* Dublin (Bek nr 801 af 26/09/2002). Fødevaredirektoratet Danmark, 1-4.

9. Anon., 2002. Salmonellosis. Annual Report on Zoonoses in Denmark 2001. The Danish Zoonosis Centre 4-17.

10. Barrington, G.M. and Parish, S.M., 2001. Bovine Neonatal Immunology. Veterinary Clinics of North America: Food Animal Practice 17, 463-476.

11. Bäumler, A.J., Tsolis, R.M. and Heffron, F., 2000. Virulence Mechanisms of Salmonella and their Genetic Basis. *In*: Wray, C., Wray, A. (Eds.), Salmonella in Domestic Animals. CABI Publishing, New York, pp. 57-72.

12. Blood, D.C. and Studdert, V.P. (Eds.), 1995. Baillière's Comprehensive Veterinary Dictionary. Baillière Tindall, W.B. Saunders, London.

13. Brooks, D.K., 1980. Inseminators as vectors of Salmonella dublin. British Medical Journal 280, 1189

14. Butler, J.E., 1983. Bovine Immunoglobulins: An augmented review. Veterinary Immunology and Immunopathology 4, 43-152.

15. Butler, W.R. and Smith, R.D., 1989. Interrelationships Between Energy Balance and Postpartum Reproductive Function in Dairy Cattle. Journal of Dairy Science 72, 767-783.

16. Cannon, R.M. and Nicholls, T.J., 2002. Relationship between sample weight, homogeneity, and sensitivity of fecal culture for *Salmonella enterica*. Journal of Veterinary Diagnostic Investigation 14, 60-62.

17. Carlsson, H.E., Lindberg, A.A. and Hammarström.S, 1972. Titration of Antibodies to Salmonella O Antigens by Enzyme- Linked Immunosorbent Assay. Infection and Immunity 6, 703-708.

18. Chambers, P.G. and Lysons, R.J., 1979. The inhibitory effect of bovine rumen fluid on *Salmonella typhimurium*. Research in Veterinary Science 26, 273-276.

19. Chaturvedi, G.C. and Sharma, V.K., 1981. Cell-mediated immunoprotection in calves immunized with rough *Salmonella dublin*. British Veterinary Journal 137, 421-430.

20. Counter, D.E. and Gibson, E.A., 1980. Salmonella dublin infection in self contained dairy herds in East Anglia: excretion at calving. Veterinary Record 107, 191-193.

21. Da Roden, L., Smith, B.P., Spier, S.J. and 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 53, 1895-1899.

22. Davies, R.H., 1997. A two year study of *Salmonella typhimurium* DT104 infection and contamination on cattle farms. Cattle Practice 5, 189-194.

23. Davies, R.H. and Wray, C., 1997. Distribution of salmonella contamination in ten animal feedmills. Veterinary Microbiology 57, 159-169.

24. Ekdahl, M.O. and Allan, C.M., 1966. Isolation of Salmonella Dublin from a sheep in New Zealand. New Zealand Veterinary Journal 14, 93

25. Enøe, C., Georgiadis, M.P. and Johnson, W.O., 2000. Estimation of sensitivity and specificity of diagnostic tests and disease prevalence when the true disease state is unknown. Preventive Veterinary Medicine 45, 61-81.

26. Evans, S.J. and Davies, R.H., 1996. Case control of multiple-resistant *Salmonella typhimurium* DT 104 infection in cattle in Great Britain. Veterinary Record 139, 557-558.

27. Fedorka-Cray, P.J., 2000. *Salmonella* Infections in Pigs. *In*: Wray, C., Wray, A. (Eds.), *Salmonella* in Domestic Animals. CABI Publishing, New York, pp. 191-207.

28. Feng, J., Li, Y., Hashad, M., Schurr, E., Gros, P., Adams, L.G. and Templeton, J.W., 1996. Bovine natural resistance associated macrophage protein 1 (Nramp1) gene. Genome Research 6, 956-964.

29. Field, H.I., 1948. A survey of bovine salmonellosis in mid and west Wales. Veterinary Journal 104, 251-339.

30. Fierer, J., 1983. Invasive Salmonella dublin Infections Associated With Drinking Raw Milk. The Western Journal of Medicine 138, 665-669.

31. Fisher, E.W., Martinez, A.A., Trainin, Z. and Meirom, R., 1976. Studies of neonatal calf diarrhoea. IV. serum and faecal immune globulins in neonatal salmonellosis. British Veterinary Journal 132, 39-48.

32. Funk, J.A., Davies, P.R. and Nichols, M.A., 2000. The effect of fecal sample weight on detection of Salmonella enterica in swine feces. Journal of Veterinary Diagnostic Investigation 12, 412-418.

33. Gibson, E.A., 1965. Reviews of Progress of Dairy Science - Diseases of Dairy Cattle. Salmonella Infection in Cattle. Journal of Dairy Research 32, 97-134.

34. Gitter, M., Wray, C., Richardson, C. and Pepper, R.T., 1978. Chronic Salmonella dublin infection in calves. British Veterinary Journal 134, 113-121.

35. Goldberg, M.B. and Rubin, R.H., 1988. The spectrum of Salmonella infection. Infectious Diseases. Clinics of North America 2, 571-598.

36. Goldsby, R.A., Kindt, T.J. and Osborne, B.A. (Eds.), 2000. Kuby Immunology, 4 Ed. W.H. Freeman and Company, New York.

37. Graat, E.A.M. and Frankena, K., 2001. Introduction To Theoretical Epidemiology. *In*: Noordhuizen, J.P.T.M., Frankena, K., Thrusfield, M.V., Graat, E.A.M. (Eds.), Application of Quantitative Methods in Veterinary Epidemiology, 2nd Ed. Wageningen Pers, Wageningen, pp. 241-259.

38. Greiner, M. and Gardner, I.A., 2000. Epidemiologic issues in the validation of veterinary diagnostic tests. Preventive Veterinary Medicine 45, 3-22.

39. Grimont, P.A.D., Grimont, F. and Bouvet, P., 2000. Taxonomy of the Genus *Salmonella*. *In*: Wray, C., Wray, A. (Eds.), Salmonella in Domestic Animals. CABI Publishing, New York, pp. 1-17.

40. Gronstol, H., Osborne, A.D. and Pethiyagoda, S., 1974. Experimental Salmonella infection in calves. 1. The effect of stress factors on the carrier state. Journal of Hygiene 72, 155-162.

41. Hall, G.A., 1975. A comparative study of liver changes produced by inoculating pregnant rats with Salmonella dublin or with its endotoxin. British Journal of Experimental Pathology 56, 216-222.

42. Hall, G.A. and Jones, P.W., 1976. An Experimental Study of *Salmonella dublin* Abortion in Cattle. British Veterinary Journal 132, 60-65.

43. Hall, G.A. and Jones, P.W., 1977. A study of the pathogenesis of experimental Salmonella dublin abortion in cattle. Journal of Comparative Pathology 87, 53-65.

44. Hardman, P.M., Wathes, C.M. and Wray, C., 1991. Transmission of salmonellae among calves penned individually. Veterinary Record 129, 327-329.

45. Helms, M., Vastrup, P., Gerner-Smidt, P. and Mølbak, K., 2003. Short and long term mortality associated with foodborne bacterial gastrointestinal infections: registry based study. British Medical Journal 326, 357-361.

46. Hinton, M., 1974. Isolation of Salmonella-Dublin from Fecal Swabs from Cattle. British Veterinary Journal 130, R31-R32.

47. Hinton, M., 1974. Salmonella-Dublin Abortion in Cattle - Studies on Clinical Aspects of Condition. British Veterinary Journal 130, 556-563.

48. Hinton, M., 1975. Salmonella-Dublin Abortion in Cattle - Incidence and Epidemiology. British Veterinary Journal 131, 94-101.

49. Holt, P.S., Wray, C. and Wray, A., 2000. Host susceptibility, resistance and immunity to Salmonella in animals. *In*: 73-87; 8 pp. of ref. Ed

50. Hoorfar, J., Feld, N.C., Schirmer, A.L., Bitsch, V. and Lind, P., 1994. Serodiagnosis of Salmonella dublin infection in Danish dairy herds using O-antigen based enzyme-linked immunosorbent assay. (Published erratum appears in Can. J. Vet. Res. 1995, 59 p. 25). Canadian Journal of Veterinary Research 58, 268-274.

51. Hoorfar, J., Wedderkopp, A. and Lind, P., 1996. Comparison between persisting anti-lipopolysaccharide antibodies and culture at postmortem in salmonella-infected cattle herds. Veterinary Microbiology 50, 81-94.

52. Houe, H., Ersbøll, A.K. and Nielsen, L.R., 2002. Nature of data for epidemiological studies. *In*: Houe, H., Ersbøll, A.K., Toft, N., Agger, J.F. (Eds.), Veterinary Epidemiology - from hypothesis to conclusion. Samfundslitteratur KVL Bogladen, Frederiksberg, pp. 25-48.

53. House, J.K., Smith, B.P., Dilling, G.W., Da Roden, L., Spier, S.J. and Picanso, J., 1991. Serologic detection of Salmonella dublin infection in cattle. *In*: Proceedings of the Ninth Annual Veterinary Medical Forum., pp. 563-566.

54. House, J.K., Smith, B.P., Dilling, G.W. and Roden, L.D., 1993. Enzyme-linked immunosorbent assay for serologic detection of *Salmonella dublin* carriers on a large dairy. American Journal of Veterinary Research 54, 1391-1399.

55. Hui, S.L. and Walter, S.D., 1980. Estimating the error rates of diagnostic tests. Biometrics 36, 167-171.

56. Jensen, A.M., Feld, N., Nielsen, B.B., Schirmer, A.L. and Madsen, E.B., 1994. Salmonella Dublin-infektion. (Eradication trial of Salmonella dublin infection in 8 dairy herds). Dansk Veterinærtidsskrift 77, 397-403.

57. Jensen, A. N. DVI, Personal Communication, 2003,

58. Jensen, C.O., 1891. Om den infektiøse Kalvediarrhoe og dens Aarsag. Maanedsskrift for Dyrlaeger 4, 140-162.

59. Kehrli, M.E., Kimura, K., Goff, J.P.S.J.R. and Nonnecke, B.J., 1999. Periparturient immunosuppression in dairy cows: nutrition and lactation effects. *In*: Wensing, Th. (Ed.), Proceedings of the 10th International Conference on Production Diseases in farm animals., pp. 41-55.

60. Kirk, J.H., Sischo, W.M., Barnett, S.C., Collar, C., Higginbotham, J. and Schultz, T., 2002. Salmonella contamination of Rubber Boots Worn on Dairies. The Bovine Practitioner 36, 11-14.

61. Konnai, S., Ohtsuka, H., Sakamoto, T., Nakaoka, Y., Kiku, Y., Fukuda, S., Koiwa, M., Takahashi, J., Taniyama, H.Y.Y., Okada, H. and Yoshino, T., 2001. Inflammatory Cytokines and Antigen-Responsive Mononuclear Cells in Peripheral Blood of Cattle Infected with *Salmonella* Takoradi. Journal of Veterinary Medical Science 63, 859-865.

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

63. Lawson, G. and Dow, C., 1966. Porcine salmonellosis. Journal of Comparative Pathology 76, 363-371.

64. Lax, A.J., Barrow, P.A., Jones, P.W. and Wallis, T.S., 1995. Current perspectives in salmonellosis. British Veterinary Journal 151, 351-377.

65. Libby, S.J., Adams, L.G., Ficht, T.A., Allen, C., Whitford, H.A., Buchmeier, N.A., Bossie, S. and Guiney, D.G., 1997. The spv genes on the Salmonella dublin virulence plasmid are required for severe enteritis and systemic infection in the natural host. Infection and Immunity 65, 1786-1792.

66. Losinger, W.C., Garber, L.P., Smith, M.A., Hurd, H.S., Biehl, L.G., Fedorka-Cray, P.J., Thomas, L.A. and Ferris, K., 1997. Management and nutritional factors associated with the detection of Salmonella sp. from cattle fecal specimens from feedlot operations in the United States. Preventive Veterinary Medicine 31, 231-244.

67. Losinger, W.C., Wells, S.J., Garber, L.P. and Hurd, H.S., 1995. Management Factors Related to *Salmonella* Shedding by Dairy Heifers. Journal of Dairy Science 78, 2464-2472.

68. Martin, S.W., Meek, A.H. and Willeberg, P. (Eds.), 1987. Veterinary Epidemiology, 1st Ed. Iowa State University Press, Ames, Iowa.

69. Mattila, T., Frost, A.J. and O'Boyle, D., 1988. The growth of salmonella in rumen fluid from cattle at slaughter. Epidemiology and Infection 101, 337-345.

70. McDonough, P.L., Fogelman, D., Shin, S.J., Brunner, M.A. and 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.

71. Meinershagen, W.A., Waldhalm, D.G. and Frank, F.W., 1970. Salmonella dublin as a cause of diarrhea and abortion in ewes. American Journal of Veterinary Research 31, 1769-1771.

72. Montagne, A., Menanteau, P., Boivin, R., Bernard, S., Lantier, F. and Lalmanach, A.-C., 2001. Cytokine gene expression in lymph node and spleen of sheep in response to *Salmonella* infection by two serotypes displaying different host specificity. Veterinary Immunology and Immunopathology 82, 257-272.

73. Morisse, J.P. and Cotte, J.P., 1994. Evaluation of some risks factors in bovine salmonellosis. Veterinary Research 25, 185-191.

74. Mortensen, A.N. and Hoorfar, J., 1999. Dyrkning af salmonella fra husdyrgødning. (Isolation of Salmonella from animal faeces). Dansk Veterinærtidsskrift 82, 707-711.

75. Munch, B., Larsen, H.E. and Nielsen, B.B., 1987. Forekomst af Salmonella i gylle fra danseke husdyrbesætninger. Dansk Veterinærtidsskrift 70, 1169-1179.

76. Muotiala, A. and Makela, P.H., 1990. The role of IFN-gamma in murine *Salmonella Typhimurium* infection. Microbial Pathogenesis 8, 135-141.

77. Murata, H. and Miyamoto, T., 1993. Bovine haptoglobin as a possible immunomodulator in the sera of transported calves. British Veterinary Journal 149, 277-283.

78. Narula, A.S., 1969. Isolation of Salmonella dublin from fowls. The Indian Veterinary Journal 46, 1103-1104.

79. Nazer, A.H.K. and Osborne, A.D., 1976. Salmonella Infection and Contamination of Veal Calves - Slaughterhouse Survey. British Veterinary Journal 132, 192-201.

80. Nazer, A.H.K. and Osborne, A.D., 1977. Experimental Salmonella Dublin Infection in Calves. British Veterinary Journal 133, 388-398.

81. Nielsen, B.B. and Vestergaard, E.-M., 1992. Use of ELISA in the eradication of Salmonella Dublin infection. *In*: Proceedings of the International Symposium on Salmonella and Salmonellosis., pp. 220-224.

82. Nielsen, S.S., 2002. Paratuberculosis in Danish dairy cattle - Interpretation of diagnostic information depending on purpose and disease stage. PhD Thesis. The Royal Veterinary and Agricultural University, Denmark.

83. Nielsen, S.S., Gröhn, Y.T., Quaas, R.L. and Agger, J.F., 2002. Paratuberculosis in Diary Cattle: Variation of the Antibody Response in Offspring Attributable to the Dam. Journal of Dairy Science 85, 406-412.

84. Nielsen, S.S., Grønbæk, C., Agger, J.F. and Houe, H., 2002. Maximum-likelihood estimation of sensitivity and specificity of ELISAs and faecal culture for diagnosis of paratuberculosis. Preventive Veterinary Medicine 53, 191-204.

85. Nielsen, S.S., Houe, H., Ersbøll, A.K. and Toft, N., 2002. Evaluating tests used in veterinary medicine. *In*: Houe, H., Ersbøll, A.K., Toft, N., Agger, J.F. (Eds.), Veterinary Epidemiology - from hypothesis to conclusion. Samfundslitteratur KVL Bogladen, Frederiksberg, pp. 129-144.

86. Olsen, J.E., Baggesen, D.L., Nielsen, B.B. and Larsen, H.E., 1990. The prevalence of plasmids in Danish bovine and human isolates of Salmonella dublin. Acta Pathologica Microbiologica Et Immunologica Scandinavica 98, 735-740.

87. Penny, C.D., Low, J.C., Nettleton, P.F., Scott, P.R., Sargison, N.D., Strachan, W.D. and Honeyman, P.C., 1996. Concurrent bovine viral diarrhoea virus and Salmonella typhimurium DT104 infection in a group of pregnant dairy heifers. Veterinary Record 138, 485-489.

88. Pesch, K.L., 1926. Meningitis durch Bacterium enteritidis (Gärtner). Zentralblatt fuer Bakteriologie 98, 22-24.

89. Peters, A.R., 1985. An Estimation of the Economic-Impact of An Outbreak of Salmonella-Dublin in A Calf Rearing Unit. Veterinary Record 117, 667-668.

90. Radostitis, O.M., Gay, C.C., Blood, D.C. and Hinchcliff, K.W. (Eds.), 2000. Veterinary Medicine, 9 Ed. Harcourt Publishers Ltd, London.

91. Richardson, A., 1973. Serological Responses of Salmonella-Dublin Carrier Cows. British Veterinary Journal 129, R53-R55.

92. Richardson, A., 1973. Transmission of Salmonella Dublin to Calves from Adult Carrier Cows. Veterinary Record 92, 112-115.

93. Richardson, A. and Fawcett, A.R., 1973. Salmonella-Dublin Infection in Calves - Value of Rectal Swabs in Diagnosis and Epidemiological Studies. British Veterinary Journal 129, 151-156.

94. Richardson, A. and Watson, W.A., 1971. A contribution to the epidemiology of *Salmonella* Dublin infection in cattle. British Veterinary Journal 127, 173-182.

95. Rings, D.M., 1985. Salmonellosis in Calves. Veterinary Clinics Of North America Food Animal Practice 1, 529-539.

96. Robertsson, J.A., 1984. Humoral antibody responses to experimental and spontaneous Salmonella infections in cattle measured by ELISA. Zentralblatt Fur Veterinarmedizin, B 31, 367-380.

97. Robertsson, J.A., Svenson, S.B., Renstrom, L.H.M. and Lindberg, A.A., 1982. Defined salmonella antigens for detection of cellular and humoral immune responses in salmonella infected calves. Research in Veterinary Science 33, 221-227.

98. Rycroft, A.N., 2000. Structure, Function and Synthesis of Surface Polysaccharides in *Salmonella*. *In*: Wray, C., Wray, A. (Eds.), Salmonella in Domestic Animals. CABI Publishing, New York, pp. 19-22.

99. Scherer, C.A. and Miller, S.I., 2001. Molecular Pathogenesis of Salmonellae. *In*: Groisman, E.A. (Ed)., Principles of Bacterial Pathogenesis. Academic Press, New York, pp. 265-333.

100. Schønheyder, H.C., Kristensen, L., Lester, A., Scheibel, J.H. and Gerner-Smidt, P., 1997. Ekstraintestinale Salmonella-infektioner i fire danske amter. Ugeskrift for Læger 159, 2082-2085.

101. Segall, T. and Lindberg, A.A., 1991. Experimental oral Salmonella dublin infection in calves: A bacteriological and pathological study. Journal of Veterinary Medicine Series B 38, 169-184.

102. Selander, R.K., Smith, N.H., Li, J., Beltran, P., Ferris, K.E., Kopecko, D.J. and Rubin, F.A., 1992. Molecular evolutionary genetics of the cattle-adapted serovar Salmonella dublin. Journal of Bacteriology 174, 3587-3592.

103. Smith, B.P., Dilling, G.W., House, J.K., Konrad, H. and Moore, N., 1995. Enzyme-linked immunosorbent assay for Salmonella serology using lipopolysaccharide antigen. Journal of Veterinary Diagnostic Investigation 7, 481-487.

104. Smith, B.P., House, J.K., Dilling, G.W., Roden, L.D. and Spier, S.J., 1992. Identification of Salmonella Dublin Carrier Cattle. *In*: Proceedings of the International symposium Salmonella and salmonellosis. Zoopôle, Ploufragan, France., pp. 225-230.

105. Smith, B.P., Oliver, D.G., Singh, P., Dilling, G., Marvin, P.A., Ram, B.P., Jang, L.S., Sharkov, N. and Orsborn, J.S., 1989. Detection of Salmonella dublin mammary gland infection in carrier cows, using an ELISA for antibody in milk or serum. American Journal of Veterinary Research 50, 1352-1360.

106. Sojka, W.J., Thomson, P.D. and Hudson, E.B., 1974. Excretion of Salmonella-Dublin by Adult Bovine Carriers. British Veterinary Journal 130, 482-488.

107. Spier, S.J., Smith, B.P., Cullor, J.S., Olander, H.J., Da Roden, L. and Dilling, G.W., 1991. Persistent Experimental Salmonella dublin Intramammary Infection in Dairy Cows. Journal of Veterinary Internal Medicine 5, 341-350.

108. Spier, S.J., Smith, B.P., Tyler, J.W., Cullor, J.S., Dilling, G.W. and Da Pfaff, L., 1990. Use of ELISA for detection of immunoglobulins G and M that recognize Salmonella dublin lipopolysaccharide for prediction of carrier status in cattle. American Journal of Veterinary Research 51, 1900-1904.

109. Steffensen, M. and Blom, J.Y., 1999. Incidence of Salmonella infections in Danish cattle herds 1992-1998. Dansk Veterinærtidsskrift 82, 966-970.

110. Steinbach, G., Dinjus, U., Gottschaldt, J., Kreutzer, B. and Staak, C., 1993. Course of infection and humoral immune reaction in calves infected orally with different salmonella serovars. Journal of Veterinary Medicine Series B 40, 515-521.

111. Steinbach, G., Koch, H., Meyer, H. and Klaus, C., 1996. Influence of prior infection on the dynamics of bacterial counts in calves experimentally infected with Salmonella dublin. Veterinary Microbiology 48, 199-206.

112. Taylor, R.J., 1973. A further assessment of the potential hazard for calves allowed to graze pasture contaminated with *Salmonella Dublin* in slurry. British Veterinary Journal 129, 354-358.

113. Taylor, R.J. and Burrows, M.R., 1971. The survival of Escherichia coli and Salmonella dublin in slurry on pasture and the infectivity of S. Dublin for grazing calves. British Veterinary Journal 127, 536-542.

114. Tizard, I.R. (Ed.), 2000. Veterinary Immunology: An Introduction, 6 Ed. W.B. Saunders Company, Philadelphia, Pennsylvania.

115. Trueman, K.F., Thomas, R.J., Mackenzie, A.R., Eaves, L.E. and Duffy, P.F., 1996. Salmonella Dublin infection in Queensland dairy cattle. Australian Veterinary Journal 74, 367-369.

116. Vaessen, M.A., Veling, J., Frankena, K., Graat, E.A. and Klunder, T., 1998. Risk Factors for *Salmonella Dublin* infection on Dairy Farms. The Veterinary Quarterly 20, 97-99.

117. van Schaik, G., Schukken, Y.H., Nielen, M., Dijkhuizen, A.A., Barkema, H.W. and Benedictus, G., 2002. Probability of and risk factors for introduction of infectious diseases into Dutch SPF dairy farms: a cohort study. Preventive Veterinary Medicine 54, 279-289.

118. Veling, J., Personal Communication, 2003, Animal Health Service, Deventer, The Netherlands.

119. Veling, J., Barkema, H.W., van der Schans, J., van Zijderveld, F. and Verhoeff, J., 2002. Herd-level diagnosis for *Salmonella enterica* subsp. *enterica* Serovar Dublin infection in bovine dairy herds. Preventive Veterinary Medicine 53, 31-42.

120. Veling, J., van Zijderveld, F.G., van Zijderveld-van Bemmel, A.M., Schukken, Y.H. and Barkema, H.W., 2001. Evaluation of Two Enzyme-Linked Immunosorbent Assays for Detecting *Salmonella enterica* subsp. *enterica* Serovar Dublin Antibodies in Bulk Milk. Clinical and Diagnostic Laboratory Immunology 8, 1049-1055.

121. Veling, J., van Zijderveld, F.G., Zijderveld-van Bemmel, A.M., Barkema, H.W. and Schukken, Y.H., 2000. Evaluation of three newly developed enzyme-linked immunosorbent assays and two agglutination tests for detecting *Salmonella enterica* subsp. *enterica* Serovar Dublin infections in dairy cattle. Journal of Clinical Microbiology 38, 4402-4407.

122. Vidal, S.M., Malo, D., Vogan, K., Skamene, E. and Gros, P., 1993. Natural resistance to infection with intracellular parasites: isolation for a candidate for *Bcg.* Cell 73, 469-485.

123. Visser, I.J.R., Veen, M., Vander Giessen, J.W.B., Peterse, D.J. and Wouda, W., 1993. Salmonella dublin in dairy herds in north Netherlands. Tijdschrift Voor Diergeneeskunde 118, 84-87.

124. Visser, S.C., Veling, J., Dijkhuizen, A.A. and Huirne, R.B.M., 1997. Economic losses due to Salmonella dublin in dairy cattle. *In*: Kristensen, A.R. (Ed.), Proceedings of the Dutch/Danish Symposium on Animal Health and Management Economics, Copenhagen., pp. 143-151.

125. Wallis, T.S., Paulin, S.M., Plested, J.S., Watson, P.R. and Jones, P.W., 1995. The Salmonella dublin virulence plasmid mediates systemic but not enteric phases of salmonellosis in cattle. Infection and Immunity 63, 2755-2761.

126. Warnick, L.D., Kanistanon, K., McDonough, P.L. and Power, L., 2003. Effect of previous antimicrobial treatment on fecal shedding of *Salmonella enterica* subsp. *enterica* serogroup B in New York dairy herds with recent clinical salmonellosis. Preventive Veterinary Medicine 56, 285-297.

127. Wathes, C.M., Zaidan, W.A.R., Pearson, G.R., Hinton, M. and Todd, N., 1988. Aerosol Infection of Calves and Mice with Salmonella- Typhimurium. Veterinary Record 123, 590-594.

128. Watson, W.A. and Watson, F.I., 1966. An outbreak of Salmonella dublin infection in chinchillas. Veterinary Record 78, 15-17.

129. Watson, P.R., Galyov, E.E., Paulin, S.M., Jones, P.W. and Wallis, T.S., 1998. Mutation of invH, but not stn, reduces Salmonella-induced enteritis in cattle. Infection and Immunity 66, 1432-1438.

130. Watson, P.R., Paulin, S.M., Bland, A.P., Jones, P.W. and Wallis, T.S., 1995. Characterization of intestinal invasion by Salmonella typhimurium and Salmonella dublin and effect of a mutation in the invH gene. Infection and Immunity 63, 2743-2754.

131. Wedderkopp, A., 1996. Salmonella incidence among cattle in Denmark [OT: Salmonellaforekomst hos kvaeg i Danmark]. Dansk Veterinærtidsskrift 79, 1002-1004.

132. Wedderkopp, A., Stroger, U., Bitsch, V. and Lind, P., 2001. Testing of bulk tank milk for Salmonella Dublin infection in Danish dairy herds. Canadian Journal of Veterinary Research 65, 15-21.

133. Wedderkopp, A., Stroger, U. and Lind, P., 2001. Salmonella dublin in Danish Dairy Herds: Frequency of Change to Positive Serological Status in Bulk Tank Milk ELISA in Relation to Serostatus of Neighbouring Farms. Acta Veterinaria Scandinavica 42, 295-301.

134. Werner, S.B., Humphrey, G.L. and Kamei, I., 1979. Association between raw milk and human Salmonella dublin infection. British Medical Journal 2, 238-241.

135. Williams, E., 1980. Veterinary surgeons as vectors of Salmonella dublin. British Medical Journal 280, 815-818.

136. Wood, P. (Ed.), 2001. Understanding Immunology. Pearson Education Limited, Essex, UK.

137. Wray, C. and Davies, R.H., 2000. Salmonella infections in cattle. *In*: Wray, C., Wray, A. (Eds.), Salmonella in Domestic Animals, 1st Ed. CABI Publishing, New York, New York State, pp. 169-190.

138. Wray, C. and Snoyenbos, G.H., 1985. Salmonella dublin infection of cattle in England and Wales: its epidemiology and control. *In*: Snoyenbos, G.H. (Ed.), Proceedings of the International Symposium on Salmonella, New Orleans., pp. 173-181.

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

140. Wray, C., Todd, N., Mclaren, I., Beedell, Y. and Rowe, B., 1990. The epidemiology of Salmonella infection of calves: The role of dealers. Epidemiology and Infection 105, 295-306.

141. Wray, C., Todd, N., McLaren, I.M. and Beedell, Y.E., 1991. The epidemiology of salmonella in calves: the role of markets and vehicles. Epidemiology and Infection 107, 521-525.

142. Wray, C., Wadsworth, Q.C., Richards, D.W. and Morgan, J.H., 1989. A three-year study of Salmonella dublin infection in a closed dairy herd. Veterinary Record 124, 532-535.

Chapter 7: Manuscripts

List of included manuscripts

Due to restrictions from the publisher of the journal in which manuscript 1 has been published, this manuscript is not present in this PDF. This manuscript can be found in:

Manuscript 1
Nielsen, L. R. & Ersboll, A. K. (2004). Age-stratified validation of an indirect *Salmonella* Dublin serum enzyme-linked immunosorbent assay for individual diagnosis in cattle. *Journal of Veterinary Diagnostic Investigation, 16,* 212-218.
http://jvdi.org/cgi/content/abstract/16/3/212

Manuscript 2, 3 and 4 have all been published, and are now available in:

Manuscript 2
L.R.Nielsen, N.Toft, & A.K.Ersbøll (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.
DOI: 10.1046/j.1365-2672.2004.02151.x

Manuscript 3
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.
DOI: 10.1016/j.prevetmed.2004.12.006

Manuscript 4
Nielsen, L. R., Schukken, Y. H., Gröhn, Y. T., & Ersbøll, A. K. (2004). Salmonella Dublin infection in dairy cattle: risk factors for becoming a carrier. *Preventive Veterinary Medicine, 65,* 47-62.
DOI: 10.1016/j.prevetmed.2004.06.010

Age stratified validation of an indirect *Salmonella* Dublin serum ELISA test for individual diagnosis in cattle

Authors: Liza Rosenbaum Nielsen and Annette Kjær Ersbøll

Location of institution: Department of Animal Science and Animal Health
The Royal Veterinary and Agricultural University
Grønnegårdsvej 8
1870 Frederiksberg C
Denmark

Short running title: *Salmonella* Dublin serum ELISA test validation

Proofs to be sent to: Liza Rosenbaum Nielsen
Department of Animal Science and Animal Health
The Royal Veterinary and Agricultural University
Grønnegårdsvej 8
1870 Frederiksberg C
Denmark
Phone: + 45 35 28 30 15
Fax: + 45 35 28 30 22
E-mail: lrn@kvl.dk

Keywords: Salmonella Dublin, ELISA, age, cattle, ROC

Accepted for publication in "Journal of Veterinary Diagnostic Investigation"

Validation of an indirect serum ELISA and a bacteriological faecal culture test for diagnosis of *Salmonella* serotype Dublin in cattle using latent class models.

Liza Rosenbaum Nielsen*, Nils Toft and Annette Kjær Ersbøll

Department of Animal Science and Animal Health,
The Royal Veterinary and Agricultural University, 1870 Frederiksberg C, Denmark

Abbreviated running headline: *Salmonella* Dublin test validation

Keywords: *Salmonella* Dublin, faecal culture, ELISA, latent class models, no gold standard.

*Corresponding author:
Liza Rosenbaum Nielsen
Department of Animal Science and Animal Health
The Royal Veterinary and Agricultural University
Grønnegårdsvej 8,
1870 Frederiksberg C
Denmark
Phone: + 45 35 28 30 15
Fax: + 45 35 28 30 22
E-mail: lrn@kvl.dk

Submitted to "Journal of Applied Microbiology"

ABSTRACT

Aims: To validate a conventional faecal bacteriological culture test and an indirect serum ELISA for detection of *S.* Dublin infected cattle. To compare the predictive values of the two tests in relation to the prevalence.

Methods and Results: A total of 4531 paired samples from cattle in 29 dairy herds were analysed for presence of *S.* Dublin bacteria in faeces and immunoglobulins directed against *S.* Dublin lipopolysaccharide in an indirect serum ELISA. Sensitivity and specificity were estimated at two ELISA cut-off values using a latent class test validation method. Stratification of data into three age groups gave significantly better estimates of test performance of the ELISA. Receiver operating characteristic (ROC) curves were constructed for comparison of overall performance of the ELISA between the three age groups. The sensitivity of the faecal culture test was low (6-14%). ELISA appeared to have a higher validity for animals aged 100-299 days of age than older or younger animals. Overall, the negative predictive value of the ELISA was 2-10 times higher than for the faecal culture test at realistic prevalence of infection in the test population.

Conclusions: The diagnostic sensitivity of the faecal culture test for detection of *S.* Dublin is poor, the specificity is 1. The superior sensitivity and negative predictive value of the serum ELISA makes this test preferable to faecal culture as an initial screening test and for certification of herds not infected with *S.* Dublin.

Significance and Impact of the Study: A quantitative estimate of the sensitivity of a faecal culture test for *S.* Dublin in a general population was provided. ELISA was shown to be an appropriate alternative test. Preferably, samples from animals aged 100-299 days of age should be used as these give the best overall performance of the ELISA. Plots of ROC curves and predictive values in relation to prevalence facilitates optimisation of the ELISA cut-off value.

INTRODUCTION

Salmonella enterica subsp. *enterica* serovar Dublin (*S.* Dublin) is a cause of disease in cattle of all ages, though the most commonly clinically affected group is calves aged 2 weeks to 3 months (Wray *et al.* 1989; Wray and Davies 2000). It is also a serious zoonosis leading to invasive infection with high mortality in humans (Fierer 1983; Helms *et al.* 2003). A special feature of *S.* Dublin is the tendency to produce persistent infections without clinical manifestation in some infected cattle – also called carriers (Richardson 1973). Such carriers host the bacteria in lymph nodes and internal organs and only periodically shed bacteria in milk and/or faeces. Results from other studies have indicated that bacteriological culture methods for detection of *S.* Dublin infected animals suffer from strong limitations with regard to sensitivity (Richardson and Fawcett 1973; Hinton 1974). One study found 3.35% of 985 faecal samples from 8 known *Salmonella* Dublin carrier cows and 17.26% of 643 faecal samples from 5 known carrier calves culture positive during a 12 month study period where the animals were tested several times a week (House *et al.* 1993). Studies including higher numbers of animals with different stages of infection are lacking. Carriers frequently have continuously high immunoglobulin levels in serum and milk (Spier *et al.* 1990; Smith *et al.* 1992). Therefore, immunoglobulin detecting ELISAs have been suggested as good alternatives to bacteriological culture for detection of *S.* Dublin infection.

An indirect ELISA was developed for use in both serum and milk to detect immunoglobulins directed against *S.* Dublin lipopolysaccharide (LPS) (Smith *et al.* 1989; Hoorfar *et al.* 1994; Hoorfar *et al.* 1995). The test can be adjusted to test different subclasses of immunoglobulins (e.g. IgG_1, IgG_2, IgM) (House *et al.* 2001). Two studies have quantified the validity of IgG detecting serum ELISAs for detection of infected animals (Veling *et al.* 2000; Nielsen and Ersbøll In press). In both these studies faecal culture was used as gold standard (i.e. an assumed perfect test for detection of infected animals). That is, the sensitivity was calculated as the proportion of ELISA positive animals out of all faecal culture positive animals, and the specificity was calculated as the proportion of ELISA negative animals out of all animals from assumed salmonella negative herds. This assumption was based on history and faecal culture results from all animals in the herds. This approach is the classic method to determine sensitivity and specificity of diagnostic tests. A major disadvantage of the classic test validation method is that it may introduce selection bias leading to potentially biased estimates of sensitivity and specificity, because the full range of infection stages in individuals is not represented in the population used for test validation.

In Denmark a national surveillance program for *S.* Dublin was initiated in October 2002. In the program all Danish cattle herds are screened by the use of ELISA response in quarterly bulk tank milk samples from dairy herds and a minimum of three yearly blood samples from non-milk producing herds. A cut-off value of 50 ODC% is used for the serum ELISA for the classification of non-milk producing herds. However, the laboratory cut-off generally used for this ELISA in Denmark is 25 ODC%, and more knowledge about the test performance at these cut-off values is needed. In the present study, the sensitivity and specificity of the serum ELISA currently used in the Danish National Surveillance program for *S.* Dublin is estimated at the two cut-off values 25 and 50 ODC%. In order to avoid biased estimates of sensitivity and specificity, and in order to obtain sensitivity and specificity estimates of the faecal culture test, the method chosen for test validation was a latent class method as described by Hui and Walter (1980). The test performances are estimated for three different age groups of cattle, and predictive values of the ELISA in relation to prevalence of infection at cut-off 25 and 50 ODC% are illustrated.

MATERIALS AND METHODS

Herds and sample collection

Herds All samples used for this study were collected from 29 dairy herds participating in a large project known as the Integrated Cattle Health and Milk Quality Project, initiated by the Danish Dairy Board in 1997 (Andersen *et al.* 2000). The herds were all situated in a region of the southern part of Jutland in Denmark. The sample collection was performed in the period March 2000 to January 2002. The herds were visited 4-5 times with approximately three months between each visit. Fifteen of the herds were considered *S.* Dublin positive based on at least one culture positive animal. Eleven herds were considered salmonella negative based on negative bacteriological culture results at all visits during the study period, and they had no history of salmonellosis prior to the study period. The remaining three herds had a few sporadic isolations of other types of salmonella bacteria (*S.* Typhimurium and *S.* Agona).

Samples At each visit to the herds the following samples were collected: Individual faecal samples from all cattle on the premises, 20 swabs from barn environment and dung pits, and blood samples from all non-lactating cattle including all dry cows in the herd. At one of the visits succeeding a finding of *S.* Dublin bacteria in 8 of the *S.* Dublin infected herds blood samples were also collected from lactating cows.

Table 1 Data used for maximum likelihood estimation of sensitivity and specificity of a serum ELISA and a faecal culture (FC) test for detection of *Salmonella* serotype Dublin infected cattle, and for estimation of prevalence of *Salmonella* serotype Dublin infected cattle of in two populations (POP1 and POP2) of dairy cattle divided into three age groups.

	Mean Age (Std)	ELISA	FC	POP1	POP2	Total
Age group 1 (0-99 days)	44 (28)					
ELISA cut-off 25 ODC%						
		-	-	397	517	914
		-	+	11	7	18
		+	-	117	99	216
		+	+	11	4	15
ELISA cut-off 50 ODC%						
		-	-	458	572	1030
		-	+	16	10	26
		+	-	56	44	100
		+	+	6	1	7
Age group 2 (100-299 days)	186 (58)					
ELISA cut-off 25 ODC%						
		-	-	237	425	662
		-	+	3	1	4
		+	-	139	115	254
		+	+	14	8	22
ELISA cut-off 50 ODC%						
		-	-	279	472	751
		-	+	5	1	6
		+	-	97	68	165
		+	+	12	8	20
Age group 3 (≥300 days)	994 (656)					
ELISA cut-off 25 ODC%						
		-	-	592	1054	1646
		-	+	5	1	6
		+	-	386	372	758
		+	+	14	2	16
ELISA cut-off 50 ODC%						
		-	-	778	1260	2038
		-	+	8	1	9
		+	-	200	166	366
		+	+	11	2	13

Blood samples were centrifuged at 3200 rpm for 20 minutes and serum was kept frozen at –18ºC at the Danish Cattle Health Laboratory (DCHL) (from 2001 Steins Laboratory) until analysis. All samples used for the test validation were collected as paired samples, including one serum sample and one faecal sample from the same animal collected on the same day.

To avoid correlation between measurements animals that were sampled more than once during the repeated visits to the 29 herds had one sample pair chosen by random selection to represent that animal.

Bacteriological culture method

Faecal samples were examined at the DCHL for the presence of salmonella bacteria using 25 g of faecal material (pool of 5 g from each of 5 animals) that was mixed in 225 mL peptone buffer and left for pre-enrichment at 37ºC for 18-24 hours. Inoculation of 0.1 ml test material onto Modified Semi-solid Rappaport Vassiliadis Medium Base (MSRV-agar) plates and 1 ml test material into 9 ml of selenite-cystine was followed by incubation for 18-24 hours at 41.5ºC. Material from the selenite-cystine tubes was inoculated on modified Brilliant-green Phenol-red Lactose Sucrose agar (BPLS-agar) plates and incubated at 37ºC for 18-24 hours. Positive test results from MSRV were inoculated onto BPLS-agar plates and confirmed using Triple Sugar Iron agar-tests and Lysine-Iron-agar tests. Serotyping and confirmation of positive isolates were conducted at the Danish Veterinary Institute (DVI). All individual samples from positive pools were cultured to identify animals shedding *S.* Dublin. This procedure will be denoted "the pool-first culture method" throughout this paper.

ELISA procedures

The serum *S.* Dublin ELISA used in this study was performed at DCHL slightly modified from a previously described ELISA method (Hoorfar *et al.* 1994). An O-antigen based *S.* Dublin LPS preparation produced at DVI was diluted 1:80,000 with 0.1 M sodium carbonate buffer pH 9.6 and used to coat microtitration plates (Polysorb Cat# 475094, Nunc, Denmark) at 4ºC for 2 days (100 µl/well). Plates were blocked using 200 µl/well phosphate buffered saline (PBS) with 1% polyvinylpyrrolidone (PVP) for 30 minutes at room temperature and then washed 3 times using PBS solution (pH 6.8) with 0.05% $Tween_{20}$. Sera were diluted 1:200 in PBS containing 1% polyvinylpyrrolidone-40T and added to the microtitration plate wells in duplicates. Known positive and negative reference sera were added in quadruplicates. The plates were incubated overnight (16-20 hours) at 4ºC, and washed 3 times. For detection of immunoglobulins, affinity purified horseradish peroxidase labelled goat anti-bovine IgG (H+L) conjugate (Cat# 14-12-06, Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) was diluted 1:6000 in PBS containing 1% PVP, added to all wells in the plate (100 µl/well), incubated for 1 hour at 37ºC and then washed 3 times. Sub-

strate and indicator solution (100 ml 0.1 M citric acid pH 5, 1,2-orthophenyldiamine dihydrochloride (Kem-En-Tec, Copenhagen, Denmark), 0.5 ml $Tween_{20}$ and 80 µl hydrogen peroxide) was added to the wells (100 µl/well) and incubated in the dark at room temperature for 10-20 minutes. The reaction was stopped by adding 100 µl/well 0.5 M H_2SO_4 to all wells when the optical density of the positive reference wells was visually evaluated to be approximately 2.000. The optical density (OD) was read at 492 nm and 620 nm as reference using an ELISA plate reader. Plates were considered valid if the 4 negative reference wells had an average OD of less than 0.300, and the 4 positive reference wells had an average OD of 1.200-2.500. An ODC%-value, which is a background corrected ratio of the test sample OD to a positive reference sample was calculated for each sample using Formula 1:

Formula 1: $$ODC\% \quad \frac{\left(\overline{OD}_{sample} - \overline{OD}_{neg\ ref}\right)}{\left(\overline{OD}_{pos\ ref} - \overline{OD}_{neg\ ref}\right)} * 100$$

where $\overline{OD}_{sample}$ is the mean value of two test wells, $\overline{OD}_{neg\ ref}$ and $\overline{OD}_{pos\ ref}$ are the mean values of four reference wells in the ELISA plates.

Statistical analysis

The performance of the two diagnostics tests were assessed by maximum likelihood (ML) estimation using the latent class model from Hui and Walter (1980). This latent class model allows for estimation of the sensitivity (Se) and the specificity (Sp) of the two tests when the true infection status of the test subjects is unknown. The model assumes that: (i) the two tests are conditionally independent given the true (but unknown) infection status; (ii) the test subjects are divided into two or more groups where the proportion of truly infected test subjects differ; (iii) the test properties are constant throughout these groups. As the initial analysis indicated that the Sp of the faecal culture (FC) test was 1, the model was modified to allow for this parameters to be fixed at 1 during the estimation. To improve the numerical stability of the estimation, the ML-estimates were obtained using an Expectation-Maximization algorithm (Dempster *et al.* 1977) implemented in the software program R®. The standard errors of the ML-estimates were obtained using the formulas given in Hui and Walter (1980) to get the elements of the Fisher Information matrix and invert this matrix to obtain the estimated asymptotic variance-covariance matrix of the ML-estimates of the parameters in the latent class model, i.e. Se_{ELISA}, Sp_{ELISA}, Se_{FC} and the prevalence of infected test subjects in each group.

Local veterinary practices were used to form the split of the data into two subpopulations. Based on this split two models were formed: Model 1, which estimated the test properties based on all the test subjects, and Model 2, where the test properties were estimated for the populations divided into three age groups: 0-99 days, 100-299 days and ≥300 days. This age grouping was selected based on results from another study (Nielsen and Ersbøll In press). These models were compared using the likelihood ratio test. Se_{ELISA}, Sp_{ELISA} and Se_{FC} were estimated at a wide range of cut-off values in the ELISA and plotted as receiver operating characteristic curves (ROCs) for the three age groups. In ROC curves the true-positive proportion (Se) and false-positive proportion (1-Sp) at different cut-off values is illustrated (Hanley and McNeil 1982; Jensen and Poulsen 1992). The use of ROC analysis facilitates selection of optimal cut-off points and comparison of the validity of different tests or test groups by comparison of the area under the ROC curves (Greiner *et al.* 2000). The area under curve (AUC) was calculated by the trapezoidal rule. Comparison of the AUCs requires access to the counts of test subjects in the cells of each of the two-by-two tables used to calculated Se and 1-Sp as described in detail by Jensen and Poulsen (1992). While the AUC can be calculated, the counts and thus the standard errors, are not available when using latent class estimation, and therefore the AUCs for the three age groups are not statistically comparable.

Negative (NPV) and positive predictive values (PPV) were calculated for the ELISA at cut-off 25 and 50 ODC% for varying prevalence in the test population using Formula 2 and 3 (Noordhuizen *et al.* 2001). Negative predictive values were calculated for the faecal culture test. The positive predictive value is always 1 for tests with Sp=1. The calculated predictive values were plotted at cut-off 25 and 50% ODC% in the ELISA.

Formula 2: $NPV = (1-P)*Sp / (P*(1-Se)+(1-P)*Sp)$

Formula 3: $PPV = P*Se / (P*Se+(1-P)*(1-Sp))$

where P is the prevalence of infected animals in the test population, Se is the estimated sensitivity and Sp is the estimated specificity of the test.

RESULTS

A total of 4531 sample pairs were available from 29 herds. *S.* Dublin was isolated from 81 of the faecal samples. The data used for the ML-estimation are shown in Table 1. According

to the likelihood ratio test result Model 2, which included age stratification of the data, gave a significantly better estimation of sensitivity and specificity than Model 1 ($p<0.0001$).

Table 2 Results from a maximum likelihood estimation of sensitivity of an ELISA (Se_{ELISA}), specificity of an ELISA (Sp_{ELISA}), sensitivity of a faecal culture test (Se_{FC}) and the prevalence (P) of *S.* Dublin infected animals in two populations (POP1 and POP2). The estimations were performed twice. Once with all animals included in the two populations (Model 1), and once with the two populations further split into three age groups (Model 2).

	Estimate (95% CI)			
	Age group1	Age group 2	Age group 3	All ages
ELISA cut-off 25 ODC%				
Se_{ELISA}	0.46 (0.38-0.53)	0.85 (0.74-0.95)	0.73 (0.65-0.80)	0.65 (0.61-0.70)
Sp_{ELISA}	0.89 (0.87-0.91)	0.88 (0.86-0.90)	0.76 (0.74-0.77)	0.82 (0.81-0.83)
Se_{FC}	0.11 (0.03-0.19)	0.12 (0.01-0.22)	0.06 (0 -0.13)	0.08 (0.04-0.13)
P_{POP1}	0.36 (0.32-0.41)	0.37 (0.32-0.42)	0.32 (0.29-0.36)	0.36 (0.34-0.39)
P_{POP2}	0.16 (0.12-0.19)	0.14 (0.10-0.18)	0.04 (0.02-0.05)	0.11 (0.09-0.12)
ELISA cut-off 50 ODC%				
Se_{ELISA}	0.21 (0.16-0.26)	0.77 (0.66-0.88)	0.59 (0.50-0.68)	0.49 (0.45-0.54)
Sp_{ELISA}	0.96 (0.93-0.98)	0.95 (0.93-0.98)	0.89 (0.88-0.91)	0.93 (0.91-0.94)
Se_{FC}	0.10 (0.04-0.16)	0.14 (0.02-0.25)	0.09 (0 -0.18)	0.10 (0.05-0.15)
P_{POP1}	0.41 (0.37-0.45)	0.32 (0.27-0.37)	0.22 (0.19-0.25)	0.29 (0.27-0.32)
P_{POP2}	0.18 (0.14-0.21)	0.12 (0.09-0.15)	0.02 (0.01-0.03)	0.09 (0.07-0.10)

Results from the estimations of test properties and population characteristics at the two ELISA cut-off values 25 and 50 ODC% are summarised in Table 2, including Se_{ELISA}, Sp_{ELISA}, Se_{FC}, estimated prevalence in the first population (P_{POP1}), estimated prevalence in the second population (P_{POP2}) with 95% confidence limits. The sensitivity of the ELISA is generally higher for age group 2 (calves 100-299 days of age) as indicated by both point estimates and 95% confidence intervals. The specificity of the ELISA is more similar between the groups and ranges from 0.76 to 0.96 depending on age group and cut-off value. The sensitivity estimates of the FC test are generally very low ranging from 0.06 to 0.14. There is a tendency for the prevalence to decrease with age in both test populations.

The ROC curves and calculated area under curves (AUCs) for the three age groups are shown together in Fig. 1. The AUC can be interpreted as the probability that the test result of a randomly drawn sample event is correct. Highly discriminatory tests have ROC curves that crowd toward the upper left corner with AUCs approaching 1. The AUC for age group 2 is clearly higher than the AUCs for age groups 1 and 3 which are more similar in size and shape.

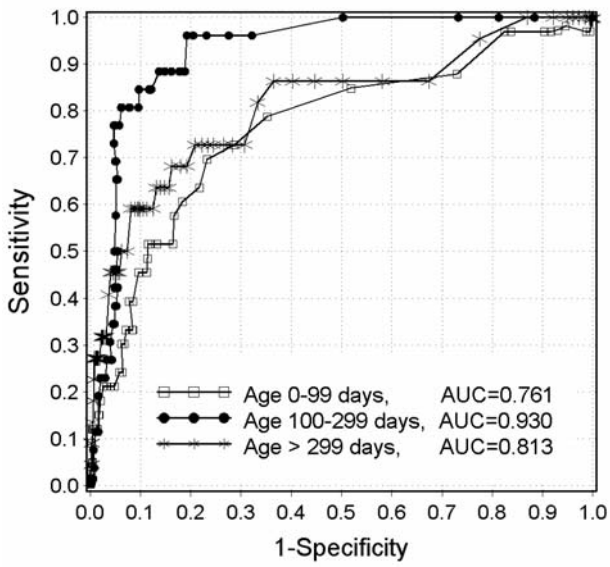


Figure 1 Receiver operating characteristics (ROC) curves for an indirect *S.* Dublin serum ELISA validated for use in three age groups of dairy cattle. The estimation of sensitivity and specificity was performed using latent class analysis. The AUCs indicate the overall performance of the test in each of the age groups.

The predictive values for the ELISA and the negative predictive value (NPV) for the FC test are shown for the three age groups at cut-off 25 and 50 ODC% in Fig. 2. The positive predictive value of the FC test is not shown because it is constant at 1. Generally, the NPV of the ELISA is highest for age group 2 and at cut-off 25%. The positive predictive value (PPV) is less influenced by age stratification and choice of cut-off value.

DISCUSSION AND CONCLUSIONS

As shown in Fig. 1 a difference was found between different age groups in performance of the serum ELISA for detection of *S.* Dublin infected cattle. It was not possible to test, if the overall performance of the ELISA was statistically different between the age groups, but the ROC curves suggest that it is easiest to optimise the sensitivity and specificity simultaneously in age group 2 (calves and young stock aged 100-299 days). The area under the ROC curve for age group 2 suggests that the ELISA test result of a randomly drawn animal

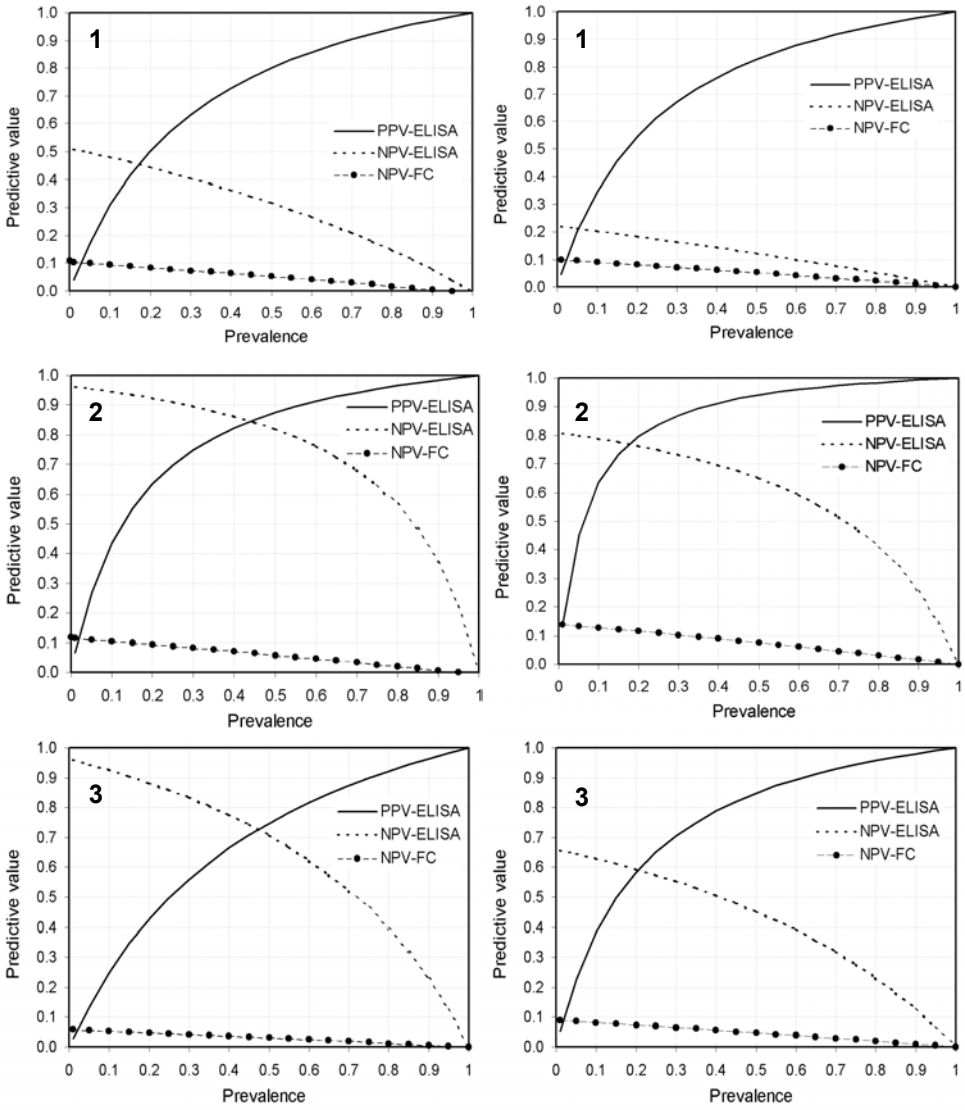


Figure 2 Positive predictive values (PPV) for a serum ELISA and negative predictive values (NPV) for a serum ELISA and faecal culture test for detection of *S.* Dublin infected animals versus prevalence in the test population. All graphs to the left show predictive values estimated at cut-off 25 ODC% in the ELISA. All graphs to the right show the predictive values estimated at cut-off 50 ODC% in the ELISA Graphs marked "1" are for age group 1 (0-99 days), those marked "2" are for age group 2 (100-299 days) and those marked "3" are for age group 3 (≥300 days).

in the positive reference population have a probability of 93% of having a higher response than a randomly drawn animal from the negative reference population (Greiner *et al.* 2000). Also, comparison of the two models with and without the split into age groups suggested that taking age into account gives a better fit of the data than ignoring the age effect on test properties. It has been shown in other studies that young calves have very little production of salmonella specific immunoglobulins until the age of 11-12 weeks leading to false negative results when using ELISA for detection of infected animals (Da Roden *et al.* 1992). Further, maternally derived antibodies from colostrum may cause false positive results in young calves. Together, this explains why the overall performance of the serum ELISA is poorer in age group 1. The ELISA also appears to have a poorer performance for cattle from the age of 300 days than for cattle 100-299 days old. This may be due to the fact that animals recovering from a *S.* Dublin infection may show false positive results in the ELISA several months after clearing the infection due to *S.* Dublin specific circulating immunoglobulins. Adult cattle may also have a higher level of cross-reacting immunoglobulins circulating in their blood, leading to a lower specificity of the ELISA (Konrad *et al.* 1994). Calves aged 100-299 days are more likely to have become infected within the last couple of months than adult cows, and are thus more likely to be truly infected at time of sampling leading to fewer false positive ELISA results.

The Se of the FC test was estimated to be between 6% and 14% with the 95% confidence limits ranging from 0% to 25% depending on the age group evaluated. The Sp was found to be 100%. Supporting our findings, House *et al.* (1993) reported that as few as 3.35% faecal samples from 8 known carrier cows and 17.26% faecal samples from 5 known carrier calves were *S.* Dublin culture positive during a 12 month study period. The FC test used in the present study could probably be improved by using direct culturing of individual faecal samples instead of the pool-first test procedure. Because bacteriological culture is an expensive method for detection of *S.* Dublin, the pool-first method was used to lower the costs while still detecting shedding animals.

The ELISA was validated at two cut-off values currently being used in practice as part of the National Surveillance program for *S.* Dublin in Denmark. Veling *et al.* (2000) evaluated an IgG_1-detecting LPS serum ELISA as a diagnostic test of acutely infected and carrier animals at several cut-off values. In that study it was found that the Se of the ELISA was highly dependent on the stage of infection (Se of 32.0% for newly infected animals and 94.4% for known active carriers of *S.* Dublin). The Sp was 99.3% at the optimal cut-off

value chosen by the authors. The calculations of test validity was based on a classic approach using faecal culture as gold standard. Nielsen and Ersbøll (In press) likewise used a classic test validation method and found a significant difference in test performance between the same three age groups used in the present study with the best overall test performance being for cattle aged 100-299 days of age. The sensitivities ranged from 21% to 82% depending on age and cut-off value (25 or 50 ODC%), which is similar to the estimates found in the present study. The specificities ranged from 93% to 99% which is higher than the estimates found in the present study (e.g. 76% to 96% depending on age group and cut-off value). Knowing that the Se of the faecal culture test is fairly poor, it is not unlikely that the Sp of the ELISA was overestimated in both studies, because infected animals and infected farms may have been misclassified as uninfected. In the present study, the Se and Sp of the ELISA were likewise found to be dependent on the age of the animal with the best performance appearing to be for animals aged 100-299 days according to the ROC curves in Fig. 1. The Sp was estimated to be at the most between 89-96%, and this was only at a fairly high cut-off (50 ODC%), where the Se was 21, 77 or 59% in age group 1, 2 and 3, respectively. Though not directly comparable, it appears that the Sp of the ELISA is overestimated and the Se is similar or slightly underestimated by the classic test validation method in relation to the presumably unbiased estimates from the latent class method.

It is not surprising to find that the predictive values of the ELISA and NPV of the FC test are highly dependent on the prevalence in the test population as shown in Fig. 2. However, it is important to realise that as prevalence decreases in the population the PPV of the ELISA decreases dramatically. At the same time, the NPV increases, but reaches a maximum dependent on the sensitivity of the test. Often, it is suggested that ROC curves are used for optimal selection of cut-off values for ELISAs. However, Fig. 2 indicates that it is also necessary to include plots of predictive values for the range of relevant prevalence in the cut-off selection process. The prevalence of *S.* Dublin infected animals in a population will most commonly be below 50%. Not only do the plots show dramatic difference in the NPV between age groups regardless of cut-off values at prevalence below 50%, they also show that only in age group 2 and 3 and only at cut-off 25 ODC% does the NPV approach 1. This is important to acknowledge if the test is intended used for certification of uninfected herds.

The use of latent class test validation methods requires that three major assumptions are fulfilled as described in the materials and methods section. In the present study, the test

validity estimates of the faecal culture test are assumed to be independent of the test validity estimates of the ELISA, given that the true state of disease is known. The assumption cannot be tested, but due to the biological and dynamical difference of analytes from the two tests (bacteria and antibodies) the assumption is assumed to be correct. All animals from all herds were included in the study (with random selection of one sample pair per animal), leaving out selection bias due to age, and the Se and Sp can be assumed to be similar in the two populations (POP1 and POP2) used for estimation in each age group. The herds were split into two populations based on which of 6 veterinary practices they belonged to. This is not likely to lead to a difference in test performance in the populations. Finally, the prevalence should be different in the two populations for the estimation to be feasible. This was also possible to fulfil with the population split used. According to Greiner and Gardner (2000), the population used for test validation should reflect the target population with regard to spectrum of disease and spectrum of other conditions. This is considered reasonable with the studied population which consists of 29 dairy herds with different levels of *S.* Dublin prevalence including herds considered free of the infection. The herds were not selected according to other criteria such as freedom from other diseases. One problem in the latent class analysis occurred with the estimation of the Se of the FC test, which should ideally be the same for all cut-off values of the ELISA. This was not entire the case though the difference in the estimates were negligible. The problem most likely occurred because the Sp was fixed to 1 for the FC test and because there were very few culture positive samples.

The conclusions of the study presented here are that latent class estimation of test sensitivity and specificity offers a useful alternative to classic test validation of diagnostic tests used to determine the *S.* Dublin infection status of cattle of all ages. The diagnostic sensitivity of the pool-first faecal culture test procedure was very low (6-14%). It is recommended to use different cut-off values in the ELISA depending on the age of the tested animal and the purpose of testing. Estimates of test sensitivity and specificity were provided at two cut-off values currently used in Danish laboratories and the Danish Surveillance Program for *S.* Dublin.

ACKNOWLEDGEMENTS

The authors thank the farmers for patient participation in intensive sample collections, the Danish Dairy Board and the Kongeå project for funding and performing the sample collection and laboratory analyses.

REFERENCES

Andersen, H.J., Aagaard, K., Skjøth, F., Rattenborg, E. and Enevoldsen, C. (2000). Integration of research, development, health promotion, and milk quality assurance in the Danish Dairy Industry. *In*: Salman, M.D., Morley, P.S., Ruch-Galie, R. (Eds.), Proceedings of the 9th Symposium of the International Society of Veterinary Epidemiology and Economics., pp. 258-260.

Da Roden, L., Smith, B.P., Spier, S.J. and 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* **53**, 1895-1899.

Dempster, A., Laird, N. and Rubin, D. (1977). Maximum likelihood from incomplete data via the EM algorithm. *Journal of Royal Statistical Society Series B* **39**, 1-38.

Fierer, J. (1983). Invasive Salmonella dublin Infections Associated With Drinking Raw Milk. *The Western Journal of Medicine* **138**, 665-669.

Greiner, M., Gardner, I.A. (2000). Epidemiologic issues in the validation of veterinary diagnostic tests. *Preventive Veterinary Medicine* **45**, 3-22.

Greiner, M., Pfeiffer, D. and Smith, R.D. (2000). Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. *Preventive Veterinary Medicine* **45**, 23-41.

Hanley, J.A., McNeil, B.J. (1982). The Meaning and Use of the Area under a Receiver Operating Characteristic (ROC) Curve. *Radiology* **143**, 29-36.

Helms, M., Vastrup, P., Gerner-Smidt, P. and Mølbak, K. (2003). Short and long term mortality associated with foodborne bacterial gastrointestinal infections: registry based study. *British Medical Journal* **326**, 357-361.

Hinton, M. (1974). Isolation of Salmonella-Dublin from Fecal Swabs from Cattle. *British Veterinary Journal* **130**, R31-R32.

Hoorfar, J., Feld, N.C., Schirmer, A.L., Bitsch, V. and Lind, P. (1994). Serodiagnosis of Salmonella dublin infection in Danish dairy herds using O-antigen based enzyme-linked immunosorbent assay. (Published erratum appears in Can. J. Vet. Res. 1995, 59 p. 25). *Canadian Journal of Veterinary Research* **58**, 268-274.

Hoorfar, J., Lind, P. and Bitsch, V. (1995). Evaluation of an O antigen enzyme-linked immunosorbent assay for screening of milk samples for Salmonella dublin infection in dairy herds. *Canadian Journal of Veterinary Research* **59**, 142-148.

House, J.K., Smith, B.P., Dilling, G.W. and Roden, L.D. (1993). Enzyme-linked immunosorbent assay for serologic detection of *Salmonella dublin* carriers on a large dairy. *American Journal of Veterinary Research* **54**, 1391-1399.

House, J.K., Smith, B.P., O'Connell, K. and VanMetre, D.C. (2001). Isotype-specific antibody responses of cattle to Salmonella Dublin lipopolysaccharide and porin following Salmonella Dublin vaccination and acute and chronic infection. *Journal of Veterinary Diagnostic Investigation* **13**, 213-218.

Hui, S.L., Walter, S.D. (1980). Estimating the Error Rates of Diagnostic Tests. *Biometrics* **36**, 167-171.

Jensen, A.L., Poulsen, J.S.D. (1992). Evaluation of Diagnostic Tests Using Relative Operating Characteristic (ROC) Curves and the Differential Positive Rate. An Example Using the Total Serum Bile Acid Concentration and the Alanine Aminotransferase Activity in the Diagnosis of Canine Hepatobiliary Diseases. *Journal of Veterinary Medicine Series A* **39**, 656-668.

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

Nielsen, L.R., Ersbøll, A.K. (In press). Age stratified validation of an indirect *Salmonella* Dublin serum ELISA for individual diagnosis in cattle. *Journal of Veterinary Diagnostic Investigation*

Noordhuizen, J.P.T.M., Frankena, K., Thrusfield, M.V. and Graat, E.A.M. (2001). *Application of Quantitative Methods in Veterinary Epidemiology*, 2nd Ed. Wageningen Pers, Wageningen.

Richardson, A. (1973). Transmission of Salmonella Dublin to Calves from Adult Carrier Cows. *Veterinary Record* **92**, 112-115.

Richardson, A., Fawcett, A.R. (1973). Salmonella-Dublin Infection in Calves - Value of Rectal Swabs in Diagnosis and Epidemiological Studies. *British Veterinary Journal* **129**, 151-156.

Smith, B.P., House, J.K., Dilling, G.W., Roden, L.D. and Spier, S.J. (1992). Identification of Salmonella Dublin Carrier Cattle. *In*: Proceedings of the International symposium Salmonella and salmonellosis. Zoopôle, Ploufragan, France., pp. 225-230.

Smith, B.P., Oliver, D.G., Singh, P., Dilling, G., Marvin, P.A., Ram, B.P., Jang, L.S., Sharkov, N. and Orsborn, J.S. (1989). Detection of Salmonella dublin mammary gland infection in carrier cows, using an ELISA for antibody in milk or serum. *American Journal of Veterinary Research* **50**, 1352-1360.

Spier, S.J., Smith, B.P., Tyler, J.W., Cullor, J.S., Dilling, G.W. and Da Pfaff, L. (1990). Use of ELISA for detection of immunoglobulins G and M that recognize Salmonella dublin lipopolysaccharide for prediction of carrier status in cattle. *American Journal of Veterinary Research* **51**, 1900-1904.

Veling, J., van Zijderveld, F.G., Zijderveld-van Bemmel, A.M., Barkema, H.W. and Schukken, Y.H. (2000). Evaluation of three newly developed enzyme-linked immunosorbent assays and two agglutination tests for detecting *Salmonella enterica* subsp. *enterica* Serovar Dublin infections in dairy cattle. *Journal of Clinical Microbiology* **38**, 4402-4407.

Wray, C., Davies, R.H. (2000). Salmonella infections in cattle. In *Salmonella in Domestic Animals* 1st Ed. CABI Publishing, New York, New York State, pp. 169-190.

Wray, C., Wadsworth, Q.C., Richards, D.W. and Morgan, J.H. (1989). A three-year study of Salmonella dublin infection in a closed dairy herd. *Veterinary Record* **124**, 532-535.

What determines the variation in the bulk tank milk response against *Salmonella* Dublin in dairy herds?

Authors: Liza Rosenbaum Nielsen and Annette Kjær Ersbøll

Location of institution: Department of Animal Science and Animal Health
The Royal Veterinary and Agricultural University
Grønnegårdsvej 8
1870 Frederiksberg C
Denmark

Short running title: Variation in *Salmonella* Dublin ELISA response in bulk tank milk

Proofs to be sent to: Liza Rosenbaum Nielsen
Department of Animal Science and Animal Health
The Royal Veterinary and Agricultural University
Grønnegårdsvej 8
1870 Frederiksberg C
Denmark
Phone: + 45 35 28 30 15
Fax: + 45 35 28 30 22
E-mail: lrn@kvl.dk

Keywords: *Salmonella* Dublin, ELISA, bulk tank milk, variance

Submitted to “Preventive Veterinary Medicine”

Abstract

Antibodies against *Salmonella enterica* subsp. *enterica* serovar Dublin (*Salmonella* Dublin) in bulk tank milk detected by enzyme-linked immunosorbent assay (ELISA) is used in certification and surveillance programs of dairy cattle herds in The Netherlands and Denmark. The sensitivity of the test has been shown to be far from perfect whereas the specificity appears to be close to 100%. More knowledge is required to understand factors causing variation in the bulk tank milk ELISA response in order to better understand the limitations and benefits of the bulk tank milk ELISA.

The objective of the present study was to determine factors influencing the bulk tank milk ELISA response. This was pursued through construction of mixed models with repeated bulk tank milk ELISA measurements in 30 Danish dairy herds, and consecutive comparison of the predictability of the models. The herds were sampled between 1 and 8 times each over a period of 3½ years. Three models were shown to perform equally well in describing the variation in the bulk tank milk ELISA response, but included different variables and therefore had different applicability for surveillance programs and intervention strategies.

In this paper we show that herd diagnosis of salmonella infection in dairy herds based on bacteriological culture lead to higher bulk tank milk response against *Salmonella* Dublin. The individual ELISA response in milk from individual cows is shown to be important for the ELISA response of bulk tank milk. However, combinations with herd size and number (or prevalence) of cows with a high ELISA response provide better models, indicating that the effect of the cow level explanatory variables on the bulk tank milk response depends on the size of the herd and the activity of the infection in the herd. The models can be used to predict the immediate effect on bulk tank milk ELISA response of intervention strategies such as culling of high responders and lowering of prevalence of infected cows in dairy herds. Predictions of the bulk tank milk ELISA response can be made with or without knowledge of the true salmonella status of the herd.

1. Introduction

Salmonella enterica subspecies *enterica* serovar Dublin (*Salmonella* Dublin) receives much attention in the cattle industry for several reasons. It is a foodborne zoonotic bacteria that causes severe invasive infections in humans – usually after consumption of milk products that have not been pasteurised properly or of insufficiently cooked meat (Fierer, 1983; Humphrey et al., 2000; Helms et al., 2003). In cattle, *Salmonella* Dublin causes economic losses in the form of disease and death among calves and young animals, as well as abortions and reproductive disorders among adult cattle, extra labour and increased veterinary expenses (Hinton, 1974; Peters, 1985; Visser et al., 1997).

The problems caused by *Salmonella* Dublin makes it desirable to control the infection both at herd level and at national level. Such control is facilitated by the use of diagnostic tests used to detect infected animals and herds. The traditionally used tests include bacteriological culture of faecal and dung pit samples and enzyme-linked immunosorbent assays (ELISAs) for detection of immunoglobulins directed against *Salmonella* Dublin in serum and milk samples (Robertsson, 1984; Hoorfar et al., 1994; Hoorfar et al., 1995; Veling et al., 2002).

Bulk tank milk ELISAs for detection of *Salmonella* Dublin infected dairy herds have been evaluated in two studies with regard to sensitivity and specificity (Wedderkopp et al., 2001; Veling et al., 2001). Whereas the specificity of a bulk tank milk ELISA detecting *Salmonella* Dublin lipopolysaccharide (LPS) was apparently high (98-100%) at the cut-off values that were selected in those studies, the herd sensitivity was far from perfect (54-76%). One study examined the effect on herd sensitivity of combining bulk tank milk ELISAs with other diagnostic tests such as culture of dung pits, drinking water, bulk milk filters and faeces from animals with current or earlier signs of salmonellosis. Also, the herd sensitivity of serology of all animals in the herd, serology of calves aged 4-6 months and serology of animals with current or earlier signs of salmonellosis was determined (Veling et al., 2002). The results showed that the combination of bulk tank milk ELISA with serology of calves aged 4-6 months produced an optimal herd sensitivity of 99%. Interestingly, the herd sensitivity of serology of all calves aged 4-6 months alone was as high as 91%. Together these findings indicate that bulk tank milk ELISA is likely to lack sensitivity when used alone, because it may not reflect the level of *Salmonella* Dublin in other groups of animals than the lactating cows. It also appears that *Salmonella* Dublin infection among the calves does not necessarily spread rapidly to the cows and produce a detectable immune response to the infection.

In October 2002 a national surveillance program for *Salmonella* Dublin was initiated by the Danish Cattle Federation and the Danish Veterinary and Food Administration. The program classifies all Danish cattle herds into three levels of *Salmonella* Dublin infection. The classification is based on bulk tank milk ELISA response against *Salmonella* Dublin LPS expressed as a percentage (ODC%) of the background corrected optical density (OD)-value to a known positive reference milk sample. Bulk tank milk samples are tested every 3 months. Herds that have a meanODC% below a cut-off value of 25 in the last four samples acquired and has not increased more than 20 ODC% since last sample are classified "Level 1" which denotes "most likely free of *Salmonella* Dublin". Herds with antibody responses in bulk tank milk above the cut-off value are classified "Level 2", and herds that have diagnosed *Salmonella* Dublin infection based on bacteriological culture are classified "Level 3" (Anon., 2002a; Anon., 2002b). In the Netherlands a bulk tank milk ELISA is used in a voluntary *Salmonella* Dublin certification program for dairy herds. The increased use of bulk tank milk ELISA response as a measure of within herd infection of *Salmonella* Dublin poses new demands for knowledge about the sources of variation of the bulk tank milk ELISA response. The objectives of the present study are to describe and analyse factors influencing the LPS ELISA response in bulk tank milk over time in herds with known *Salmonella* Dublin infection status. The initial hypothesis was that the variation in bulk tank milk is mainly explained by the antibody level in the lactating cows and the salmonella status of the herd. The analyses were based on 30 Danish dairy herds that were sampled repeatedly over a period of 3½ years.

2. Materials and methods

2.1. Herd selection

The sample collection was part of a large project known as the "Integrated Cattle Health and Milk Quality Project" initiated by the Danish Dairy Board in 1997 (Andersen et al., 2000). All herds were located in the so-called Kongeå-region in the Southern part of Jutland, where *Salmonella* Dublin is considered endemic. A total of 35 herds participated on a voluntary basis in repeated sampling of all cows. The sampling was performed by milk quality advisors from the Danish Dairy Board, who collected individual milk samples during repeated visits to each of the herds every 3 months in the period March 2000 to January 2002. In addition, individual milk samples were collected monthly through the milk quality control scheme from September 2001 to September 2003. The infection status of the herds was determined by extensive sampling including individual faecal samples from all animals, environmental swabs from all barn sections and the dung pits to attempt to isolate

salmonella bacteria from these samples. Bulk tank milk samples were collected routinely on a monthly basis through the milk quality control scheme.

Whenever a bulk tank milk sample was collected within a period of 3 days before or after individual milk samples from all cows, this set of samples (bulk tank milk and individual samples) was included in the data set for analysis. A total of 30 herds were represented with between 1 and 8 sets of samples each. One herd with a serious outbreak of *Salmonella* Typhimurium DT170 was excluded from the study, because the diagnosis of this outbreak was clear, and it would not have been misinterpreted as a *Salmonella* Dublin infected herd. The last four of the 35 herds did not have any bulk milk samples collected within 3 days from the individual milk samples. In total, 83 sets of samples originating from the 30 herds were used for the analysis.

Milk yield data were collected from the Danish Cattle Database. For every individual milk sample, the milk yield was recorded no more than 20 days before or after the sample date. If no milk yield was recorded in this 40 days period, the animals were assumed not to contribute milk to the bulk tank milk.

2.2. Variables

The herd infection status (HDST) was determined as salmonella positive, if salmonella bacteria were found at any point in time during the study period. In 11 herds, *Salmonella* Dublin was the only salmonella bacteria found. In 3 herds, both *Salmonella* Dublin and other types of salmonella bacteria were found. In 3 herds, *Salmonella* Typhimurium was the only bacteria isolated. In 2 herds other or non-typable types of salmonella bacteria were found. In 11 herds, no salmonella bacteria were found during the study period.

The mean yield corrected OD (meanYCOD) in the herd was calculated based on individual yield corrected OD (YCOD) values from each cow. The YCOD is a value indicating the contribution of antibodies to the bulk tank milk from the individual cow, and it was calculated as the individual milk yield (kg) multiplied with the ODC% measured in the individual milk sample, divided by the total milk production (kg) in the herd on the same day. The relationships between the individual YCOD and individual milk yield and the individual ODC% are illustrated in Fig. 1 for 6425 sample events from all lactating cows in the 30 herds. Variables tested in the analysis were:

At herd level:

- Herd infection status (HDST) (salmonella positive or negative)

At herd sample date level (variables all measured on the day of sampling of all individual milk samples):

- Mean ELISA response of all individual milk samples (meanODC%)
- Prevalence of cows above 25 ODC% or faecal culture positive (AP)
- Mean yield corrected OD (meanYCOD)
- Prevalence of high responders (cows with above 80 ODC% in the individual milk sample (Prev80))
- Number of high responders (NHR)
- Herd size (HS) measured as number of cows (both lactating and dry cows)

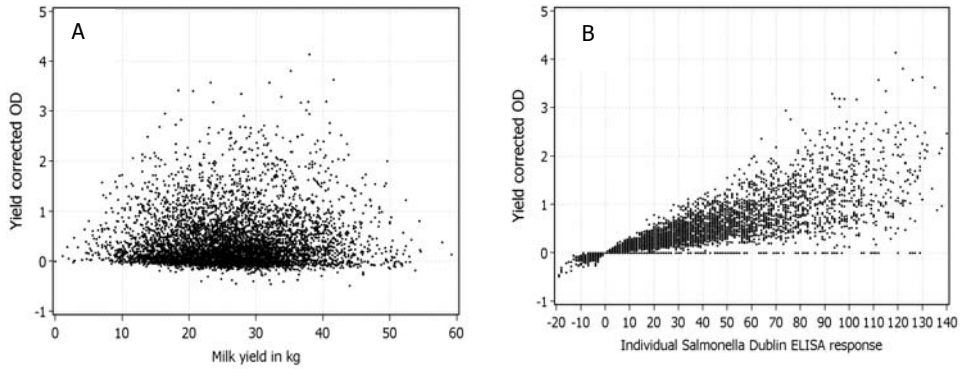


Figure 1 The relation between the individual yield corrected OD and A: daily milk yield in kg, and B: ELISA response (ODC%) in individual milk samples.

2.3 Laboratory procedures

The ELISA method used for the individual and bulk milk samples is described in detail elsewhere (Nielsen and Ersbøll, In press). In short, microtitration plates were coated with a *Salmonella* Dublin LPS antigen produced at the Danish Veterinary Institute (DVI). Sample milk was added undiluted to the microtitration plate wells in duplicates. Known positive and negative reference milk was added in quadruplicates. Following incubation, bound immunoglobulins were detected by a affinity purified horseradish peroxidase labelled goat anti-bovine IgG (H+L) conjugate. Substrate and indicator solution were added and incubated in the dark for approximately 15 minutes. The reaction was stopped when the optical density (OD) of the positive reference wells was evaluated to be approximately 2,000. The ODC%-value, which is a background corrected ratio of the test sample OD to a positive reference sample was calculated for each sample as follows:

$$ODC\% = \frac{(\overline{OD}_{sample} - \overline{OD}_{neg\ ref})}{(\overline{OD}_{pos\ ref} - \overline{OD}_{neg\ ref})} * 100\%$$

where $\overline{OD}_{sample}$ is the mean value of two test wells, and $\overline{OD}_{neg\ ref}$ and $\overline{OD}_{pos\ ref}$ are the mean values of four reference wells in the ELISA plates.

All bacteriological culture methods used in the study were standard procedures for detection of salmonella bacteria performed at Steins Laboratory and the Danish Veterinary Institute (DVI). The methods are described in detail in Nielsen and Ersbøll (In press).

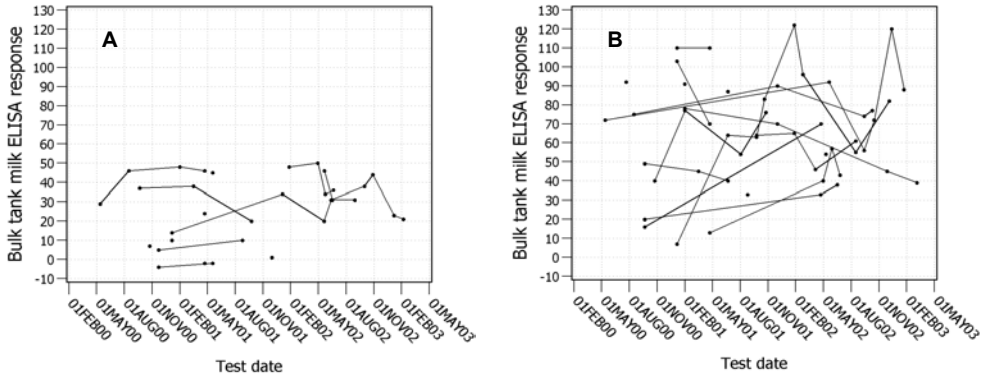


Figure 2 Distribution of bulk tank milk ELISA response against *Salmonella* Dublin LPS in herds classified as A: salmonella negative and B: salmonella positive. The herds were sampled between 1 and 8 times each.

2.4. Statistical analysis

The statistical analysis consisted of construction of multivariable mixed models with repeated measurements of bulk tank milk ELISA response against *Salmonella* Dublin LPS using the MIXED procedure in SAS® version 8.2. The models were build by backward elimination of non-significant fixed effects and their two-way interactions. The criteria for keeping fixed effects in the models were $p<0.05$ and equal or better model fit as assessed by the likelihood ratio test and change in Akaike's Information Criterion (AIC) for nested models. If a fixed effect was borderline significant when kept in the model but lead to a better fit, the effect was kept in the model. The correlation structure used for the repeated measurements was a spatial power structure. Herd salmonella status was taken as a binary variable (yes/no), and all other variables were regarded as continuous. Linear relations

between the bulk tank milk ELISA response and the continuous explanatory variables were evaluated using scatter plots. Strong multicollinearity was found between the meanODC%, meanYCOD and AP which were consequently not tested simultaneously in the models. The same was the case for the NHR and Prev80 leaving six possible combinations of the variables to be tested in the full models. Ten-fold cross validation of each of the six final models and comparison of the overall predictive error of the models was performed as suggested by Weisberg (1985) and illustrated by Emborg et al. (2001). For each of the ten cross validations, a model using approximately 90% of the observations was used to predict the last 10% of the dataset. The overall model prediction error was calculated as the sum of the squared differences between observed and predicted values for each model, divided by the number of observations. Because all six models had similar prediction errors, final model selection was based on simplicity and assumed application value. The percentage of variation explained by the models was calculated as the ratio: $(R_e - R_m)/ R_e$ where R_e is the estimated residual variance of the empty model and R_m is the estimated residual variance of the model in question.

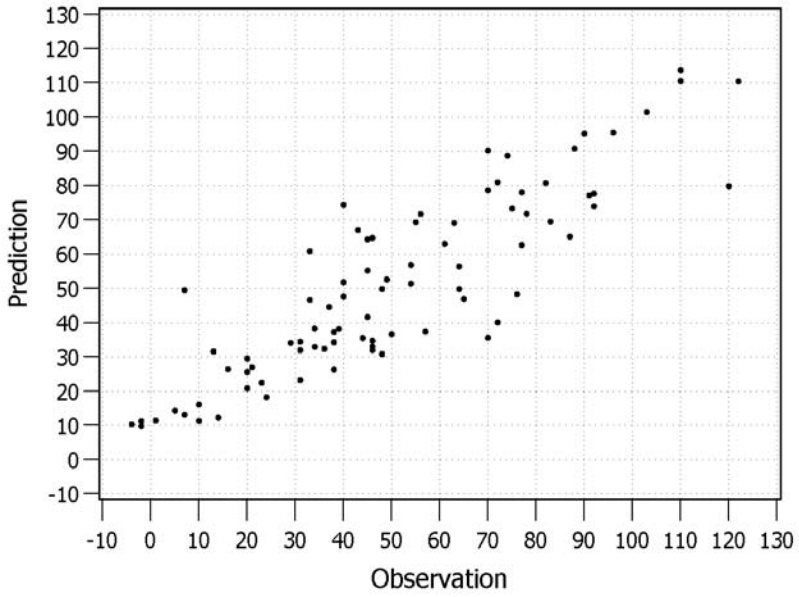


Figure 3 Ten-fold cross validation of Model 1. Predicted values are plotted against observed values.

3. Results

Descriptive statistics of bulk tank milk ELISA response and the fixed effects are shown in Table 1. In Fig. 2 the distribution of measured bulk tank milk ELISA response in herds with and without salmonella is shown. Six final models were found to perform equally well according to the predictive errors and model fit statistics. The results of three of these final models are shown in Table 2.

Model 1 includes the herd salmonella status, the apparent prevalence of infected cows and the number of high responders. The model explained 77% of the variation in the bulk tank milk ELISA response. The predicted values are plotted against observed values for Model 1 in Fig. 3. The predictions were very similar for Models 2 and 3.

Table 1 Descriptive statistics of bulk tank milk ELISA response and explanatory variables tested in models for bulk tank milk ELISA response in 30 dairy herds (83 observations).

	Bulk tank ELISA response (ODC%)	Herd size (# cows)	Mean-ODC%	Apparent prevalence (%)	Prevalence (%) of high responders[a]	Number of high responders	Mean-YCOD[b]
Maximum	122	144	82	94	55	35	1.35
Q3	72	88	37	54	14	11	0.5
Median	46	64	26	40	6	5	0.34
Q1	31	56	17	25	2	1	0.2
Minimum	-4	32	-2	0	0	0	-0.05

[a] High responders are cows with an individual ELISA response >80 ODC%

[b] Mean individual yield corrected OD-contribution from all cows in the herd

In Fig. 4. the predicted bulk tank milk ELISA response is illustrated at different values of explanatory variables included in Model 1. The number of high responders (NHR) in known salmonella infected herds were generally higher than in assumed non-infected herds.

Model 2 includes the herd salmonella status (HDST), the meanODC% and herd size (HS). These variables are easy accessible, except for bacteriologically diagnosed herd salmonella status, which may require intensive sampling to be obtained correctly. The model explained 78% of the variation in the bulk tank milk ELISA response. In Fig. 5 the predicted outcome is plotted as a function of the meanODC% at three different herd sizes. Herd size was borderline significant, however removal of the effect from the model lead to a significantly poorer fit of the model.

Model 3 includes the meanYCOD, the prevalence of high responders (Prev80), herd size, and the interaction between meanYCOD and Prev80. Examples of predicted bulk tank milk ELISA response based on Model 3 for herds with different meanYCODs and different

herd sizes are shown in Fig. 6. This model also explained 77% of the variation in the bulk tank milk ELISA response.

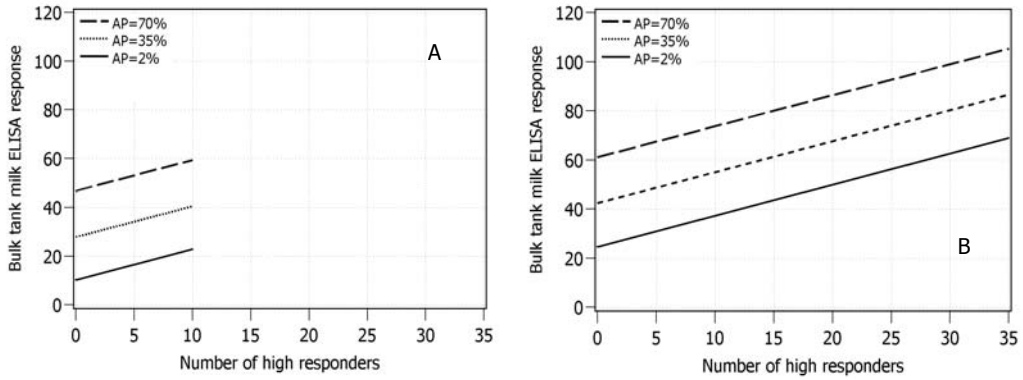


Figure 4 Predicted bulk tank milk ELISA response against *Salmonella* Dublin as a function of the number of high responders in dairy herds with no salmonella infection (A) or with salmonella infection (B). The predictions are shown for a low, a mid and the high apparent prevalence values measured in the two groups. Model 1 was used for the prediction.

The 3 resulting models are as follows:

Model 1: $Y_{ijk} = \mu + HDST_i + \beta_1 \times AP_{ijk} + \beta_2 \times NHR_{ijk} + \varepsilon_{ijk}$

Model 2: $Y_{ijk} = \mu + HDST_i + \beta_3 \times meanODC\%_{ijk} + \beta_4 \times HS_{ijk} + \varepsilon_{ijk}$

Model 3: $Y_{ijk} = \mu + \beta_5 \times meanYCOD_{ijk} + \beta_6 \times Prev80_{ijk} + \beta_7 \times HS_{ijk} + \beta_8 \times meanYCOD_{ijk} \times Prev80_{ijk} + \varepsilon_{ijk}$

Where

Y_{ijk} is the predicted bulk tank milk ELISA response in ODC% herds with herd infection status i (i=negative, positive), sample date j (j=1….8) and herd k (k=1…..30).

μ is the overall mean (intercept)

β are slopes

$meanYCOD_{ijk}$ is the mean yield corrected OD contribution to the bulk tank milk

$Prev80_{ijk}$ is the prevalence of high responders (cows with >80 ODC% in individual milk ELISA)

HS_{ijk} is herd size measured as number of cows

$HDST_i$ is herd status (salmonella negative or salmonella positive)

$MeanODC\%_{ijk}$ is the mean individual ELISA response against *Salmonella* Dublin

AP_{ijk} is the apparent prevalence of *Salmonella* Dublin infected animals

NHR_{ijk} is the number of high responders

ε_{ijk} is the random error

Table 2 Result of the final models describing explanatory variables and covariance parameters of bulk tank milk ELISA response for *Salmonella* Dublin

Effects	Estimates	S.E.	Test value[c]	*P*
MODEL 1				
Fixed				
Intercept	23.6376	5.2799	4.48	< 0.0001
Herd status				
Salmonella negative	-14.4259	4.5836	-3.15	0.0039
Salmonella positive	0			
Apparent prevalence (AP) [a]	53.4454	14.6468	3.65	0.0006
Number of high responders (NHR)	1.2621	0.4383	2.88	0.0058
Covariance				
Herd	0.9823[b]	0.007133	137.7	< 0.0001
MODEL 2				
Fixed				
Intercept	9.6267	8.3652	1.15	0.2595
Herd status				
Salmonella negative	-11.8117	4.8184	-2.45	0.0207
Salmonella positive	0			
Mean-ODC%	1.1840	0.1121	10.56	< 0.0001
Herd size (# cows)	0.1494	0.07555	1.98	0.0534
Covariance				
Herd	0.9797[b]	0.007851	124.79	< 0.0001
MODEL 3				
Fixed				
Intercept	-17.3235	8.03	-2.16	0.0394
Mean-YCOD	65.0055	15.3115	4.25	< 0.0001
Prevalence of high responders (Prev80)	162.97	64.6808	2.52	0.0151
Herd size (#cows)	0.4277	42.3396	-2.4	< 0.0001
Mean-YCOD*Prev80	-101.57	0.08703	4.91	0.0203
Covariance				
Herd	0.9818[b]	0.007301	134.47	< 0.0001

[a] Range 0-1

[b] Covariance estimate per day between samples within herd

[c] The test value is based on a t-test for the fixed effects and a χ^2-test for the covariance parameter

Discussion

In a study by Wedderkopp (2000) the maximum sensitivity of the bulk tank milk *Salmonella* Dublin ELISA was 76% when the specificity was 100%. The validation was based on 52 herds from different parts of Denmark. The reference test used for determination of the *Salmonella* Dublin status of the herds was a similar ELISA used on individual milk samples from the herds. If more than one animal tested positive in the individual milk ELISA, the herd was classified positive for *Salmonella* Dublin. The true *Salmonella* Dublin status of the herds as such was unknown. In the present study we found a strong relation between individual milk ELISA response, the apparent prevalence and bulk tank milk ELISA response. This suggests that using individual ELISA response as reference test for validation of the bulk tank milk ELISA is not ideal.

Veling et al. (2001) evaluated the validity of two different bulk tank milk ELISAs for detection of *Salmonella* Dublin infected herds, an LPS detecting ELISA and a flagellar antigen detecting ELISA. Sensitivity and specificity were estimated based on herds with known infection status (based on bacteriology and history). At the optimal cut-off OD-value of 0.2 in the LPS ELISA the sensitivity was 54%, and the specificity was 98% when estimated using the Dutch control herds and 100% when using the Swedish control herds. In other words, the truly *Salmonella* Dublin infected herds were only detected in 54% of the cases when a bulk tank milk LPS ELISA was used for screening. Veling et al. (2001) also found that 62% of the variance in the OD value of the bulk tank milk LPS ELISA could be explained by the combination of the percentage of seropositive lactating cows in the herd and the mean $\log_{10}$ serum antibody titre for all lactating cows in the herd. This supports the results based on Models 1 and 2 in the present study, as in Model 1 the percentage of seropositive cows (apparent prevalence) and in Model 2 the meanODC% are highly significant. However, Model 1 suggests that if the number of high responders in the herd is included in the model with the apparent prevalence, the variation is much better accounted for. Model 2 suggests that when including the herd size, the herd variation is better accounted for. As illustrated in Fig. 5 small herds in general have a lower bulk tank milk ELISA response than larger herds with the same mean individual ELISA response in milk, though the effect is only borderline significant in the model. In Fig. 5 it is also shown that herds with salmonella infection are likely to have a higher bulk tank milk response than herds without salmonella, even when the meanODC% is the same.

Comparison of Models 2 and 3 suggests that the yield of cows with a high antibody level and the prevalence of high responders explains some of the effect in Model 3 that is explained by the salmonella status in the herd in Model 2. The two effects, NHR and mean-

YCOD, appear to explain the herd infection status indirectly. It was not possible to differentiate the bulk tank milk response of herds infected with *Salmonella* Dublin compared other types of salmonella with the available data (data not shown). This may be due to some degree of cross-reaction in the *Salmonella* Dublin ELISA with O-antigens from other types of salmonella bacteria, in particular *Salmonella* Typhimurium in cattle (Konrad et al., 1994). It may also simply be a question of too small a sample size of herds with infections other than *Salmonella* Dublin in the present study.

Models 1 and 2 indicate that infection with salmonella bacteria significantly increases the bulk tank milk ELISA response against *Salmonella* Dublin LPS, even when controlling for the mean ELISA response of cows in the herd and the number of cows in the herd (Model 2), or when controlling for the apparent prevalence and number of high responders (Model 1).

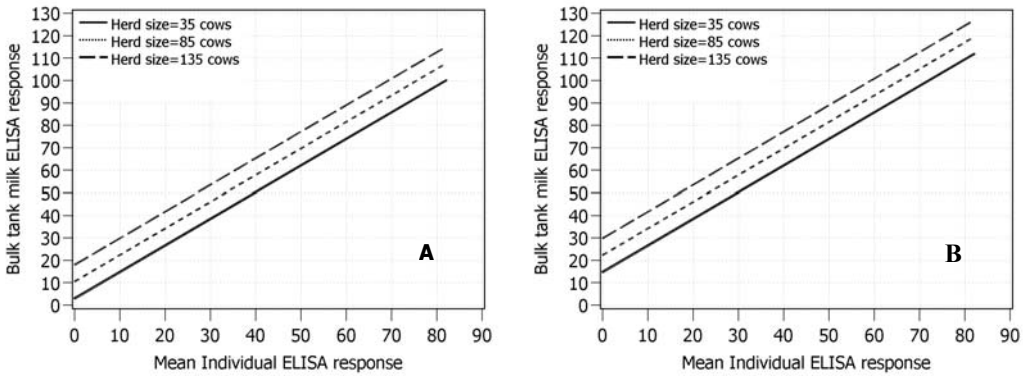


Figure 5 Predicted bulk tank milk ELISA response against *Salmonella* Dublin as a function of the mean individual ELISA response in milk from all cows in dairy herds with 35, 85 and 135 cows using Model 2 in dairy herds without diagnosed salmonella infection (A) and with known salmonella infection (B).

Model 3 showed that knowledge about the infection status of the herd is not necessary to explain most of the variation. This may be useful in situations where the herd status with regard to salmonella infection is unknown. Further, this model includes a variable that is dependent on the milk yield of cows in the herd. As illustrated in Fig. 1, the individual YCOD of a cow is mainly determined by the ELISA response from individual milk but high yielding cows contribute more antibodies to the bulk tank milk when infected. It could be hypothesized that high yielding cows are also more likely to have a high individual ELISA response in infected herds, because they may shed bacteria more frequently or have an active long term infection leading to a higher YCOD, if they become infected. This

hypothesis is supported by other studies indicating that cows with high levels of antibodies are more likely to shed bacteria (House et al., 1993), and that cows subject to stress and negative energy balance are more likely to become infected with salmonella and to become carriers of *Salmonella* Dublin (Spier et al., 1991; Morisse and Cotte, 1994; Nielsen et al.). This hypothesis could not be tested with the available data in the present study, but are highly relevant for future studies.

The models can be used in practice to determine relevant intervention strategies for the cows in herds that cannot be certified free of *Salmonella* Dublin infection. As illustrated in Figs. 4 and 5, Model 1 and Model 2 can be used when the infection status of the herd is known, for instance in herds that had a diagnosed outbreak of *Salmonella* Dublin within the last 1-2 years. The figures show that herds that are heavily infected – i.e. herds with a high apparent prevalence (or high mean ELISA response) have to both decrease the prevalence of infected cows and the number of high responders in order to be able to reach a bulk tank milk ELISA response close to the cut-off value used in the Danish National Surveillance system. This can be obtained by culling high responders (short term intervention strategy) and preventing transmission pathways between animals in a herd (long term intervention strategy). An option for use when the infection status of the herd is unknown is shown in Fig. 6. The meanYCOD of the herd can be calculated if access to individual milk yield on the day of sampling is available. The prediction of Model 3 shows that in herds with high meanYCODs it is not sufficient to cull high responders to obtain a low bulk tank milk response, whereas herds with a low meanYCOD will fairly easy be able to lower the bulk tank milk ELISA response to below the desired cut-off.

Conclusions

The objective of the study was to determine factors influencing the variation of bulk tank milk ELISA response against *Salmonella* Dublin. The results showed that several different models could explain the variation in the bulk tank milk ELISA response equally well. Three models with different applicability were illustrated. Salmonella infection in a dairy herd was shown to lead to higher bulk tank milk response against *Salmonella* Dublin. The individual ELISA response in milk from cows were important for the level of bulk tank milk when measured as a mean ODC%, apparent prevalence and a yield corrected OD contribution to the bulk tank milk (YCOD). However, combinations with herd size and prevalence or number of high responders increased the predictability of the bulk tank milk ELISA response. This indicates that the factors evaluated influence the bulk tank milk response differently dependent on the size of the herd and the activity of the infection in the herd.

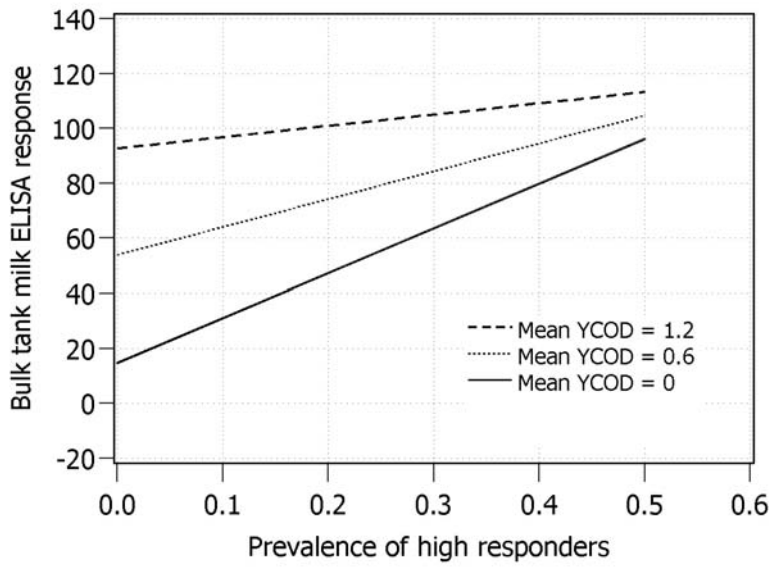


Figure 6 Predicted bulk tank milk ELISA response against *Salmonella* Dublin as a function of the prevalence of high responders in dairy herds with 75 cows. The predictions are shown for low, medium and high mean yield corrected optical density contributions (Mean YCOD) using Model 3.

Acknowledgements

The authors wish to thank the farmers for participating in the Kongeå-project which provided the data for the study. The work was funded by the Danish Dairy Board and the Kongeå-project.

References

Andersen, H.J., Aagaard, K., Skjøth, F., Rattenborg, E. and Enevoldsen, C., 2000. Integration of research, development, health promotion, and milk quality assurance in the Danish Dairy Industry. In: Salman, M.D., Morley, P.S., Ruch-Galie, R. (Eds.), Proceedings of the 9th Symposium of the International Society of Veterinary Epidemiology and Economics., pp. 258-260.

Anon., 2002a. Anvisning i fastsættelse af Salmonella Dublin niveau (CIR nr 9451 af 04/10/2002, Forskrift vedr. BEK Nr. 801 af 26/09/2002). Fødevaredirektoratet Danmark, 1-12.

Anon., 2002b. Bekendtgørelse om *Salmonella* Dublin (Bek nr 801 af 26/09/2002). Fødevaredirektoratet Danmark, 1-4.

Emborg, H., Ersbøll, A.K., Heuer, O.E. and Wegener, H.C., 2001. The effect of discontinuing the use of antimicrobial growth promoters on the productivity in the Danish broiler production. Prev. Vet. Med. 50, 53-70.

Fierer, J., 1983. Invasive *Salmonella* dublin Infections Associated With Drinking Raw Milk. West. J. Med. 138, 665-669.

Helms, M., Vastrup, P., Gerner-Smidt, P. and Mølbak, K., 2003. Short and long term mortality associated with foodborne bacterial gastrointestinal infections: registry based study. Br. Med. J. 326, 357-361.

Hinton, M., 1974. *Salmonella*-Dublin Abortion in Cattle - Studies on Clinical Aspects of Condition. Br. Vet. J. 130, 556-563.

Hoorfar, J., Feld, N.C., Schirmer, A.L., Bitsch, V. and Lind, P., 1994. Serodiagnosis of *Salmonella* dublin infection in Danish dairy herds using O-antigen based enzyme-linked immunosorbent assay. (Published erratum appears in Can. J. Vet. Res. 1995, 59 p. 25). Can. J. Vet. Res. 58, 268-274.

Hoorfar, J., Lind, P. and Bitsch, V., 1995. Evaluation of an O antigen enzyme-linked immunosorbent assay for screening of milk samples for Salmonella dublin infection in dairy herds. Can. J. Vet. Res. 59, 142-148.

House, J.K., Smith, B.P., Dilling, G.W. and Roden, L.D., 1993. Enzyme-linked immunosorbent assay for serologic detection of *Salmonella* dublin carriers on a large dairy. Am. J. Vet. Res. 54, 1391-1399.

Humphrey, T., Wray, C. and Wray, A., 2000. Public-health aspects of Salmonella infection. In: Wray, C., Wray, A. (Eds.), Salmonella in Domestic Animals. CABI Publishing, New York, New York State, pp. 245-263.

Konrad, H., Smith, B.P., Dilling, G.W. and House, J.K., 1994. Production of Salmonella serogroup D (O9)-specific enzyme-linked immunosorbent assay antigen. Am. J. Vet. Res. 55, 1647-1651.

Morisse, J.P., Cotte, J.P., 1994. Evaluation of some risks factors in bovine salmonellosis. Vet. Res. 25, 185-191.

Nielsen, L.R., Ersbøll, A.K., In press. Age stratified validation of an indirect *Salmonella* Dublin serum ELISA for individual diagnosis in cattle. Journal of Veterinary Diagnostic Investigation

Nielsen, L.R., Schukken, Y.H., Grohn, Y.T. and Ersbøll, A.K. *Salmonella* Dublin infection in dairy cattle: Risk factors for becoming a carrier. Unpublished.

Peters, A.R., 1985. An Estimation of the Economic-Impact of An Outbreak of Salmonella-Dublin in A Calf Rearing Unit. Vet. Rec. 117, 667-668.

Robertsson, J.A., 1984. Humoral antibody responses to experimental and spontaneous Salmonella infections in cattle measured by ELISA. Zentralbl. Veterinarmed. B. 31, 367-380.

Spier, S.J., Smith, B.P., Cullor, J.S., Olander, H.J., Da Roden, L. and Dilling, G.W., 1991. Persistent Experimental *Salmonella* dublin Intramammary Infection in Dairy Cows. J. Vet. Int. Med. 5, 341-350.

Veling, J., Barkema, H.W., van der Schans, J., van Zijderveld, F. and Verhoeff, J., 2002. Herd-level diagnosis for *Salmonella enterica* subsp. enterica Serovar Dublin infection in bovine dairy herds. Prev. Vet. Med. 53, 31-42.

Veling, J., van Zijderveld, F.G., van Zijderveld-van Bemmel, A.M., Schukken, Y.H. and Barkema, H.W., 2001. Evaluation of Two Enzyme-Linked Immunosorbent Assays for Detecting Salmonella enterica subsp. enterica Serovar Dublin Antibodies in Bulk Milk. Clin. Diag. Lab. Imm. 8, 1049-1055.

Visser, S.C., Veling, J., Dijkhuizen, A.A. and Huirne, R.B.M., 1997. Economic losses due to Salmonella dublin in dairy cattle. In: Proceedings of the Dutch/Danish Symposium on Animal Health and Management Economics, Copenhagen., pp. 143-151.

Wedderkopp, A., 2000. Application of Serological Assay for Screening of Salmonella Dublin Infection in Dairy Herds.

Wedderkopp, A., Stroger, U., Bitsch, V. and Lind, P., 2001. Testing of bulk tank milk for Salmonella Dublin infection in Danish dairy herds. Can. J. Vet. Res. 65, 15-21.

Weisberg, S., 1985. Applied Linear Regression, 2 Ed. John Wiley & Sons, St. Paul.

Salmonella Dublin infection in dairy cattle: Risk factors for becoming a carrier

L.R. Nielsen[1*], Y.H. Schukken[2], Y.T. Gröhn[2] and A.K. Ersbøll[1].

[1]Department of Animal Science and Animal Health,
The Royal Veterinary and Agricultural University, 1870 Frederiksberg C, Denmark
[2]Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca NY 14853, USA

Keywords: *Salmonella* Dublin, carrier, risk factor, within herd prevalence

*Corresponding author:
Liza Rosenbaum Nielsen
Department of Animal Science and Animal Health
The Royal Veterinary and Agricultural University
Grønnegårdsvej 8,
1870 Frederiksberg C
Denmark
Phone: + 45 35 28 30 15
Fax: + 45 35 28 30 22
e-mail: lrn@kvl.dk

Submitted to "Preventive Veterinary Medicine"

Abstract

Longterm *Salmonella* Dublin carrier animals that carry the pathogen in lymph nodes and internal organs and periodically shed bacteria through feces or milk, contribute considerably to transmission of the pathogen within infected herds. Thus, it is of great interest to reduce the number of new carrier animals in cattle herds over time. An observational field study was performed to evaluate factors affecting the risk that dairy cattle become carrier animals upon infection with *Salmonella* Dublin.

Based on repeated sampling, animals in 12 Danish dairy herds were categorized according to course of infection. Some of the animals could be categorized as either carriers (n=157) or transiently infected (n=87). The infection dates for each of these animals were estimated from fecal excretion and antibody responses. The relationship between the course of infection (carrier or transiently infected) and risk factors at estimated time of infection were analyzed using a random-effect multilevel, multivariable logistic regression model.

The animals with the highest risk of becoming carriers when infected with *Salmonella* Dublin were heifers infected between the age of 1 year and first calving, and cows infected around time of calving. Season and prevalence of potential shedders in the herd at time of infection changed the risk of becoming a carrier animal. The risk was higher in the first two quarters of the year (late Winter to Spring), and the risk was highest when the prevalence of potential shedders in the herd was low. The risk of becoming a carrier animal upon infection was higher in some herds than others. The herds with the highest risk of carrier development were herds with clinical disease outbreaks during the study period. The results show that the increased stress load on heifers and cows around time of calving and during disease periods increase the risk of becoming a carrier upon infection with *Salmonella* Dublin. These findings are useful for future control strategies against *Salmonella* Dublin, because they show the importance of optimized calving management and management of heifers, and because they show that even when the herd prevalence is low, carriers are still being produced leading to persistence of the infection in herds. The results raise new questions to the pathogenesis of *Salmonella* Dublin with regard to the immune mechanisms involved in development of the carrier state in cattle upon infection with low infection doses.

Introduction

Salmonella enterica subspecies *enterica* serovar Dublin (*Salmonella* Dublin) is a cause of concern in the Danish cattle industry for several reasons. It is a potential zoonosis causing severe invasive infections in susceptible humans (Fierer, 1983; Schønheyder et al., 1997; Humphrey et al., 2000). In the cattle industry *Salmonella* Dublin causes economic losses in the form of death among calves and young animals, abortions and reproductive disorders among adult cattle, and extra labor and increased veterinary expenses (Hinton, 1974b; Peters, 1985; Visser et al., 1997).

Official registrations of clinical disease outbreaks probably underestimate the prevalence of *Salmonella* Dublin as infection can be latent in herds with no or rare clinical symptoms (Wray and Davies, 2000). The registered number of *Salmonella* Dublin isolations from clinical outbreaks varied from 52 to 143 herds in Denmark during the years 1992 to 1998 (Steffensen and Blom, 1999). Compared to a national seroprevalence of 15-20% positive herds reported in a study of Danish dairy cattle in 1994-1996 (equivalent to approximately 2000-2300 infected herds on a national basis), this suggests that *Salmonella* Dublin infections are more common in Danish cattle than estimated from outbreak recordings (Wedderkopp et al., 2001).

A major issue for control of *Salmonella* Dublin infections in cattle herds is the tendency of this bacteria to produce longterm carriers that periodically shed bacteria to the environment and contribute to the spread of infection within herds (Wray and Snoyenbos, 1985; Smith et al., 1989; Veling et al., 2000). Carriers are animals that after initial infection continue to carry the infection in lymph nodes and internal organs, and either continuously or intermittently shed high numbers of *Salmonella* Dublin bacteria through feces and/or milk. Several studies have investigated the performance of laboratory methods to detect such carriers in herds. One study suggested that a positive immunoglobulin-G (IgG) response measured by an enzyme linked immunosorbent assay (ELISA) in two serum samples collected at 60-day intervals made the best prediction of a carrier state (Spier et al., 1990). The carriers selected were active carriers that were excreting almost continuously, so the conclusions from this study may not apply to less obvious carriers that only excrete intermittently. Smith et al. (1989) showed that antibody response of transiently infected animals may take several months to decrease to undetectable levels using ELISAs. A third study showed that 3 serum samples collected over a period of 120 days could differentiate between carriers and recovered animals based on IgG ELISA response (Smith et al., 1992).

Carriers are known to shed bacteria intermittently. It has been suggested that carriers may start to shed bacteria during periods of stress such as calving (Counter and Gibson, 1980) and transportation (Gronstol et al., 1974; Wray et al., 1991), whereas others (Smith et al., 1989) did not find increased shedding around calving compared to the rest of the lactation period. Risk factors for occurrence of *Salmonella* Dublin infection in cattle herds have been reported to be: Trade of animals, herd size, liver fluke infestation, concurrent BVD-infection, certain feeding strategies and climate (Wray and Roeder, 1987; Morisse and Cotte, 1994; Losinger et al., 1997; Vaessen et al., 1998; Steffensen and Blom, 1999). However, with the exception of (House et al., 1991) who reported that neonatal *Salmonella* Dublin-septicemia in calves may lead to systemic and mammary gland carriers of the bacteria as heifers, and Spier et al. (1991) who found that corticosteroid injections 7 weeks after inoculation of *Salmonella* Dublin into the mammary gland lead to recrudescence of bacterial excretion and lymphatic spread of the bacteria to the regional lymph nodes, little is known about why some animals become carriers when they become infected with *Salmonella* Dublin.

The objective of the present study was to examine the effect of animal related factors such as age, parity and lactation stage, environment related factors such as exposure level of *Salmonella* Dublin (measured as prevalence of potential shedders in the herds and age groups) and the effect of season on the risk of becoming a carrier animal as opposed to recovering when infected with *Salmonella* Dublin. The overall study hypothesis was that reduced immune capacity of young calves and animals under increased stress load such as around the time of calving may increase the risk of animals becoming carriers instead of recovering, when they are infected with *Salmonella* Dublin. The analyses were based on data from a longitudinal field study in 12 Danish dairy herds that were endemically infected with *Salmonella* Dublin during the study period.

Materials and methods

Data collection

The present study was part of a Danish project, the Integrated Cattle Health and Milk Quality Project, initiated by the Danish Dairy Board in 1997 (Andersen et al., 2000). Out of 249 dairy herds in the project region known as the Kongeå-region in the southern part of Jutland, 111 herds participated in projects about infectious diseases on a voluntary basis.

For the present study 12 herds (11 Holstein-Friesian breed and 1 Jersey breed) were selected for participation based on mid to high bulk tank milk measurements for antibodies against *Salmonella* Dublin or knowledge of *Salmonella* Dublin infection in the herds. They also had to be willing to allow fairly extensive sampling in their herds. The main part of sample collection consisted of 5 visits to each herd with approximately 3 months between visits. At each farm visit, rectal fecal samples were collected from all animals, heparin stabilized blood samples were collected from all non-lactating animals and milk samples were collected from all lactating animals on the premises. If animals were out on pasture they were not sampled. Five of the 12 herds were selected to participate in a related project involving more frequent and intensive sampling around time of calving. Herd 7 experienced a severe outbreak of *Salmonella* Dublin among adult cattle in September 2001, and was known to have had *Salmonella* Dublin infection on the premises about 5 years earlier. This herd was included in the sample collection from October 2001. In 6 herds, monthly milk samples were collected from all cows through the milk recording scheme from Fall 2001 to Fall 2002. Herd 1 also had clinical disease consistent with salmonellosis in the cows, and Herds 2 and 5 had outbreaks of clinical disease consistent with salmonellosis among calves and young stock during the study period.

Laboratory diagnostics

Bacterial cultures performed before October 1, 2001 were generally carried out on pooled fecal samples (pool of 5 g from each of 5 animals) and confirmed at individual level if a pool was found positive. After October 2001 pooling of fecal samples was stopped and all fecal samples were analyzed individually (25 g per animal). Several studies report low sensitivity of bacterial culture methods to detect *Salmonella* Dublin infected animals (Richardson and Fawcett, 1973; Hinton, 1974a), but the methods have not been evaluated in detail for this infection. In the present study fecal cultures were used in combination with serum or milk ELISAs except for calves below the age of 50 days, where maternally derived antibodies may cause a high number of false positives when interpreting ELISA results. The sensitivity of pooled fecal cultures was assumed to be 25%, and the sensitivity of individual fecal cultures was assumed to be 50% in this age group. The sensitivity of fecal cultures is likely to be lower for older animals.

ELISAs were used to evaluate the level of immunoglobulins directed against *Salmonella* Dublin surface-lipopolysaccharides in serum and milk. An ODC%, which is a background

corrected ratio of the test sample OD to a known positive reference sample was calculated for each sample as follows:

$$ODC\% = \frac{(\overline{OD}_{sample} - \overline{OD}_{neg\ ref})}{(\overline{OD}_{pos\ ref} - \overline{OD}_{neg\ ref})} * 100\%$$

where $\overline{OD}_{sample}$ is the mean value of two test wells, $\overline{OD}_{neg\ ref}$ and $\overline{OD}_{pos\ ref}$ are the mean values of four reference wells in the ELISA plates.

The serum ELISA has been described and validated in detail elsewhere (Nielsen and Ersbøll, In press). Test validation was performed for the milk ELISA using culture positive animals for sensitivity calculations and animals from culture negative herds for specificity calculations, and the test validity was determined to be a sensitivity of 50% (95% CI: 33-67%) and a specificity of 93% (95% CI: 91-95%) for every sampling event with a cut-off value of 48 ODC% in the milk ELISA. For the serum ELISA a cut-off value of 35 ODC% was chosen resulting in a sensitivity of 65% (95% CI: 55-75%) and a specificity of 97% (95% CI: 96-98%) for every sampling event. These cut-off values were chosen so that the two ELISAs had the most even performance. The milk ELISA, as it was performed for this study, had lower validity at all cut-off values than the serum ELISA. If an animal seroconverted between two sampling dates the sample immediately after seroconversion was denoted positive even if the cut-off value of 35 or 48 ODC% had not been reached yet. Seroconversion was defined as a rise in ODC% of more than 25 in the serum ELISA and more than 30 in the milk ELISA. Since most animals were tested using one ELISA and one bacterial culture test, parallel sensitivities and specificities were calculated as described by (Martin et al., 1987), and adjusted according to the added seroconversion criteria. The parallel test validity estimates used for further calculations are shown in Table 1.

Table 1 Apparent prevalences and estimated true prevalences (TP) in %, and the parallel sensitivities and specificities of the diagnostic tests used to estimate the true prevalence of *Salmonella* Dublin infected animals in five different age groups in the last quarter of year 2000. The last columns show the estimated herd prevalence in the 12 study herds.

Age groups	Calves 0-50 days		Calves 51-160 days		Young stock 161-400 days		Heifers >400 days		Cows		Herd	
	AP	TP[a]	AP	TP	AP	TP	AP	TP	AP	TP	AP	TP
Herds												
1	0	4	0	2	0	4	6	2	28	34	14	13
2	0	21	0	7	0	3	-[c]	-[c]	19	17	11	8
3	0	7	0	21	48	53	-[c]	-[c]	70	100	55	76
4	0	8	57	63	37	39	0	3	34	45	28	35
5	0	9	47	51	92	100	77	87	15	12	48	66
6	0	9	0	6	7	4	18	17	34	45	23	27
7[b]	0	7	88	100	72	79	73	81	59	80	59	75
8	0	6	65	73	70	78	49	54	43	62	47	64
9	0	7	0	7	16	14	21	20	22	24	17	19
10	0	12	0	8	9	6	-[c]	-[c]	35	48	22	26
11	0	7	35	37	78	89	28	28	32	43	43	58
12	0	21	25	25	67	75	-[c]	-[c]	32	42	37	49
Estimated test validity												
Sensitivity[d]	25%		88%		88%		88%		64%		71%	
Sensitivity[e]	50%		90%		90%		90%		68%		77%	
Specificity	100%		96%		96%		96%		91%		95%	

[a] In the true prevalence estimation only bacterial culture sensitivity was used to adjust the apparent prevalence for this age group

[b] As this herd had not yet entered the study in 2000, the estimates shown from this herd are from the last quarter of year 2001, when the herd entered the study after a *Salmonella* Dublin outbreak that started among the adult cows in the herd in September 2001.

[c] Animals on pasture. Number of tested animals is too low to estimate prevalence in that age group.

[d] Parallel test sensitivity before October 1, 2001 when pooled fecal samples were being used for bacteriological cultures.

[e] Parallel test sensitivity after October 1, 2001 when individual fecal samples were being used for bacteriological cultures.

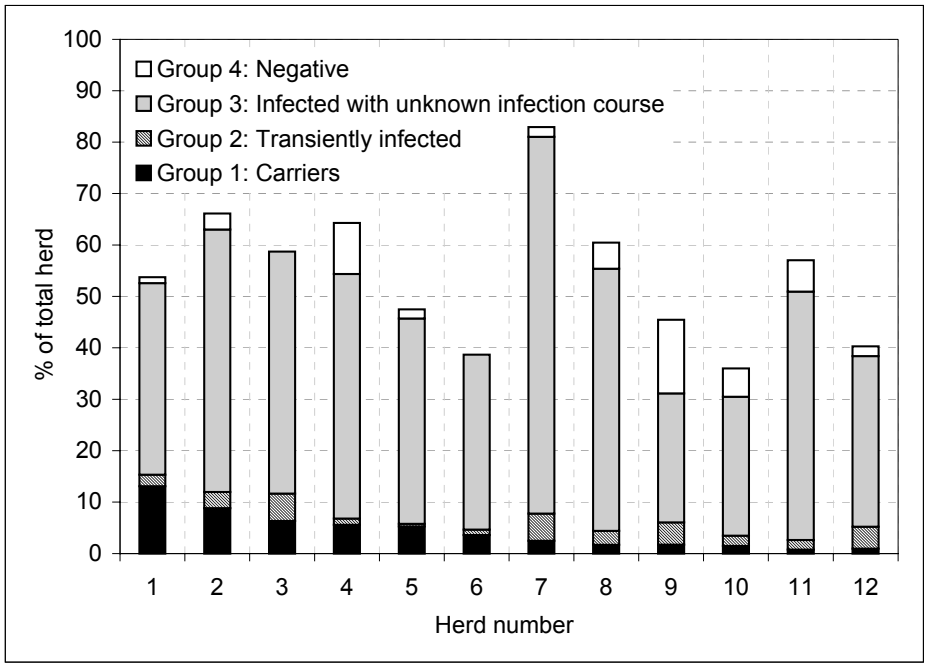


Fig. 1 Distribution of animals in 4 categories of *Salmonella* Dublin infection course in 12 study herds. The rest of the animals in the herds did not have sufficient data to be categorized.

Categorization according to course of infection

A retrospective case-control study was used in which carrier animals were considered cases, and transiently infected animals constituted the control group of animals. All animals sampled were categorized according to the course of *Salmonella* Dublin infection. The categorization was performed as follows: For an animal to be categorized as either carrier, transiently infected or negative it had to be sampled at least 4 times for a period of at least 270 days. The first sample had to have a negative laboratory result in order to estimate the infection date. Animals were defined as being *Salmonella* Dublin carrier animals (Group 1 in Fig. 1) if they tested positive for a period of more than 240 days with the last sample being positive. The length of the period was required to be at least 240 days in order to minimize misclassification of transiently infected animals as carriers. 157 animals were categorized as carriers. Animals with at least one positive laboratory test result, testing positive for less than 90 days with the last sample being negative were categorized as transiently infected (Group 2 in Fig. 1). The 90 days were chosen as the threshold for transiently infected animals based on the results of two other studies (Spier et al., 1990). 87 animals were categorized as transiently infected. Animals that did not have sufficient data to be

categorized in Group 1 or 2, but that were found positive in at least one sample were categorized as infected with unknown course of infection (Group 3 in Fig. 1). Animals that were sampled at least 4 times with a maximum of 122 days between each sample for a period of more than 270 days, and that did not test positive in any sample were categorized as negative (Group 4 in Fig. 1). Animals with too few samples or too short a sampling period did not add new information and are not shown here. This group of animals consists mainly of new animals entering the herds (imported or newborn animals) or animals leaving the herd for slaughter or trade during the sampling period. Fig.1 shows the distribution of animals in Groups 1-4 in each of the 12 study herds.

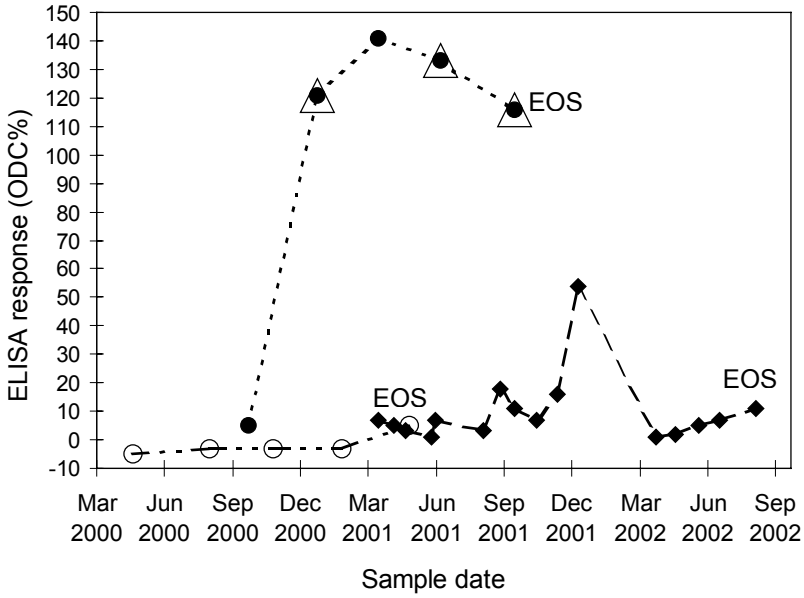


Fig. 2. Examples of laboratory results from animals with 3 different courses of *Salmonella* Dublin infection. All animals had low ELISA response at the first sample date. The carrier (- - • - -) was seropositive on all 4 consecutive sample dates and fecal culture positive (Δ) on 3 sample dates and was estimated to be infected on October 18, 2000. The transiently infected animal (—♦—) that was assumed to be positive for less than 90 days was estimated to be infected on November 18, 2001. The negative animal (- - — O - -) was test negative at all samples taken in the study period. EOS denotes end of study for each animal.

Estimation of infection date

Estimation of date of infection was based on knowledge about humoral antibody response and fecal excretion of *Salmonella* Dublin from experimental studies. The overall conclu-

sions from such studies are that fecal excretion may be intermittent in carrier animals (Smith et al., 1989), and in newly infected animals excretion often drops to undetectable levels within the first 2-4 weeks after infection (Robertsson, 1984). Humoral antibody response, such as a rise in immunoglobulins, reaches measurable levels within approximately 1-3 weeks in most infected animals (Robertsson, 1984). However, some animals do not respond to infection and part of the reason may be an age-related lack of ability to produce immunoglobulins in young calves (Da Roden et al., 1992). For Groups 1 and 2, the date of infection was estimated as the date halfway between the first possible infection date (7 days prior to the last negative test result), and the last possible infection date (14 days prior to the first positive test result). However, if the animal tested positive for *Salmonella* Dublin only based on a positive fecal culture with no concurrent immunological response measured at the first positive sample date, the infection date was set to be the date of the first positive sample. Fig. 2 illustrates examples of typical sampling routines, laboratory test results, categorization of infection course and infection dates for Groups 1, 2 and 4 in the study population.

Herd and age group prevalence estimates

The prevalence of *Salmonella* Dublin infected animals (potential shedders) was estimated for five age groups in each herd and for each herd across age groups. Calves aged 0-50 days were kept in either single calf huts or single indoor pens. Calves 51-160 days old were usually kept in common areas of 4-8 animals with straw bedding. Young stock 161-400 days old were usually kept in common areas of 4-15 animals with slatted floors or deep straw. Heifers were kept in either common areas with slatted floors or tie stalls in the cow barn, and cows in either loose housing systems with slatted floors (6 herds) or tie stalls (6 herds). The apparent prevalence (AP) was calculated as the number of test positive animals out of all animals tested in each quarter of a year for each age group and the whole herd. If an animal was tested more than once in the same quarter, one sample date was chosen by random selection to represent that animal for prevalence calculations. The true prevalence estimate (TP) was obtained by adjusting AP by the parallel sensitivity and specificity of the laboratory diagnoses (Smith, 1995). If no test positive animals were found, the estimate of the true prevalence was calculated as half the maximum possible number of infected animals (Martin et al., 1987), due to the imperfect nature of the diagnostic tests.

Table 2 Frequency distribution of variables for carriers and transiently infected animals. All variables relate to the estimated date of infection of every individual animal.

Infection status	Carriers n=157		Transiently infected n=87	
Categorical variables	No.	%	No.	%
Age and Time from calving (Age&TFC)				
Calves:	13	8.3	18	20.7
Heifers:	41	26.1	2	2.3
Close to 1st calving[a]:	17	10.8	6	6.9
Cows after 1st calving[b]:	39	24.8	23	26.4
Close to 2nd and higher calving[c]:	9	5.7	1	1.2
Cows after 2nd calving[d]:	38	24.2	37	42.5
Season				
January - March	25	15.9	5	5.8
April - June	53	33.8	28	32.2
July - September	22	14.0	17	19.5
October - December	57	36.3	37	42.5
Continuous variables	Mean	Se	Mean	Se
True prevalence in age group (in %)	31.4	20.2	43.1	24.3
True herd prevalence (in %)	29.9	16.8	44.7	17.9

Season, age, parity and lactation stage

Season was defined as the quarter of the year of the estimated date of infection (e.g. January-March, April-June, July-September and October-December). Calving and production data were obtained from the Danish Cattle Database: At the estimated date of infection the time from calving (TFC) was calculated as the number of days between the estimated infection date and the last calving date, unless the animal had not calved before, in which case it would be a negative number indicating number of days before 1st calving. If a cow was less than 70 days before the next calving at time of infection, TFC was calculated as time to next calving. A new categorical variable (Age&TFC) was constructed from a mix of the age, parity and TFC of each animal at date of infection. The following coding was used:

1) Calves below one year of age
2) Heifers above one year and up to 70 days before 1st calving
3) 1st parity cows and heifers in the period 1st calving ± 70 days
4) 1st parity cows after 70 days into 1st lactation and up to 70 days before 2nd calving

5) 2nd or higher parity cows in the period calving ± 70 days
6) 2nd or higher parity cows after 70 days into lactation and up to 70 days before next calving

Statistical analysis

A multilevel, multivariable logistic regression model was built using backward elimination. To adjust for intraherd correlation, herd was included as a random effect in the model. The binary outcome was the infection course, carrier (Group 1) or transiently infected (Group 2). Risk factors tested were related to the estimated infection date of the individual animals in the study. The risk factors tested in the model were: Age&TFC, season, herd prevalence, age group prevalence and all two-way interactions. Estimation of the different parameters was performed using the GLIMMIX macro in SAS® version 8.2. The deviance was evaluated for goodness-of-fit in relation to the degrees of freedom in the model. The fit of the final model was checked using Pearsons' residuals and partial residual plots for the full model and each variable (Collett, 1991).

Results

True prevalence estimates of Salmonella Dublin

The apparent and true prevalence estimates calculated for each quarter varied over time within and between herds. The calculated prevalence estimates for the last quarter of year 2000 are shown in Table 1. The results showed a large variation in estimated true prevalence between age groups within each herd, ranging from 7% to 100% in different age groups. Also a large variation in herd prevalence ranging from 8% to 76% was found. Fig. 3 shows the fluctuation of the true prevalence within each age group and across age groups during the study period in Herd 1. This herd was thought to be initially infected with *Salmonella* Dublin by import of carrier animals in 1990, when the herd had a severe outbreak of salmonellosis among calves. From December 2000 to February 2001 the herd had an increase in reproductive disorders and clinical disease among cows and heifers. The causal factor was thought to be poor quality of stored silage. After the disease outbreak there was an increase in the number of *Salmonella* Dublin excreting animals, and the bacteria was found in most age groups during all of year 2001. The overall prevalence of potential shedders in the 12 herds varied between quarters of each year, i.e. the mean prevalence within all 12 herds was 27.0% in the 2nd quarter of 2000 but 51.3% in the 2nd quarter of 2001. A similar trend was seen for other quarters of the two years with 2001 generally having the highest prevalence of potential shedders.

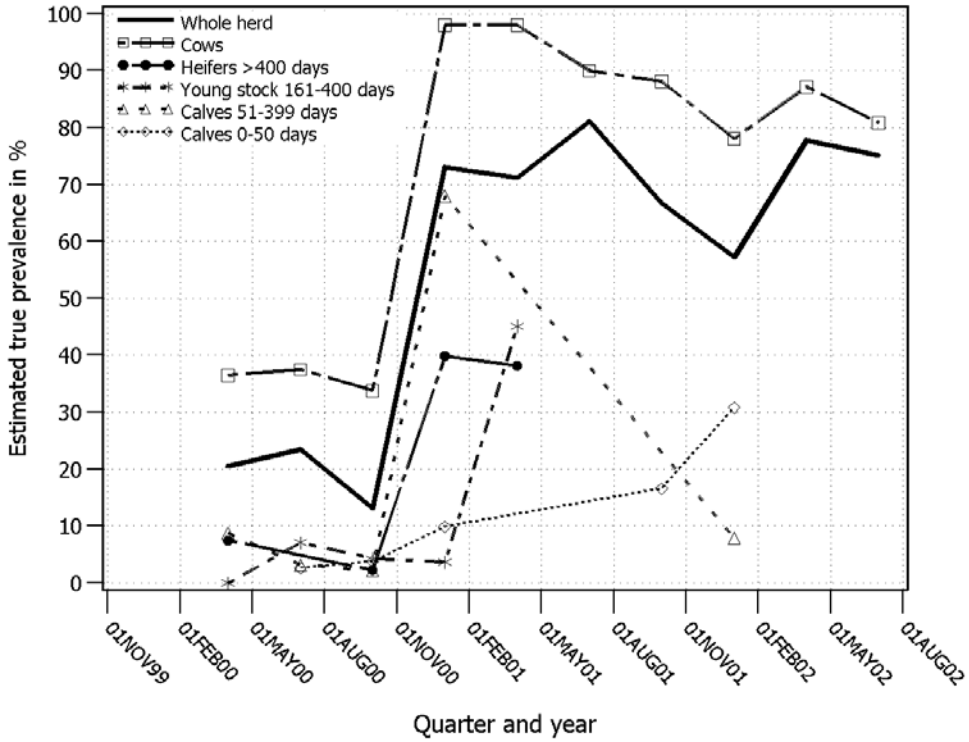


Fig. 3 Fluctuation of true prevalence estimates over time in Herd 1. This herd, which was endemically infected with *Salmonella* Dublin, experienced an outbreak of disease among cows and heifers in the period December 2000 to February 2001. A rise in the number of *Salmonella* Dublin excreting animals was seen after the disease outbreak.

Frequency distribution of single variables

Table 2 shows the distribution of all variables. The variables age-and-time from calving (Age&TFC), season, age group prevalence and herd prevalence of infected animals all appear to have an influence on the risk of becoming a carrier upon infection. Fig. 4 shows the frequency distribution of carriers and transiently infected calves and heifers in relation to the age of the animals tested.

Resulting model

Risk factors associated with the course of infection of *Salmonella* Dublin were season (borderline significant, $p=0.057$), herd prevalence ($p<0.001$) and Age&TFC group ($p=0.027$) (Table 3) at time of infection. Herd was included in the model as a random effect. It was

borderline significant (p=0.07) according to the Wald test value with this data. It was considered important to keep herd as a random effect in the model due to the hierarchical data structure, and due to a better model fit of the data when herd was included as a random effect. Residual plots showed a few outliers but no trend in the plots. The model was tested without the outliers, and this did not change parameter estimates or statistical inferences made from the model to any noteworthy extent. Together, these finding suggest the model was well fit. The interaction between Age&TFC and season was not possible to evaluate due to lack of data in some sub-groups. None of the other 2-way interactions tested were statistically significant at a significance level of 0.05. The predicted risks of the Age&Time-groups for becoming carrier animals upon infection with *Salmonella* Dublin in the first quarter of the year are shown in Fig. 5. The figure illustrates the effect of herd as a random effect.

The resulting model was found to be as follows:

$$\text{Logit}(p_{ijkl}) = \mu + AT_i + S_j + \beta * TP_{kl} + H_k + \varepsilon_{ijkl}$$

where

p_{ijkl} is the probability for becoming a carrier for cow l infected Age&Time-group i in herd k and season j.

μ is the general mean

AT_i is the fixed effect of age and time from calving, i=1….6

S_j is the fixed effect of season, j=1..4

β is the slope

TP_{kl} is the true prevalence estimate in herd k at estimated infection time for cow l

H_k is the random effect of herd, k=1….12

ε_{ijkl} is the random error

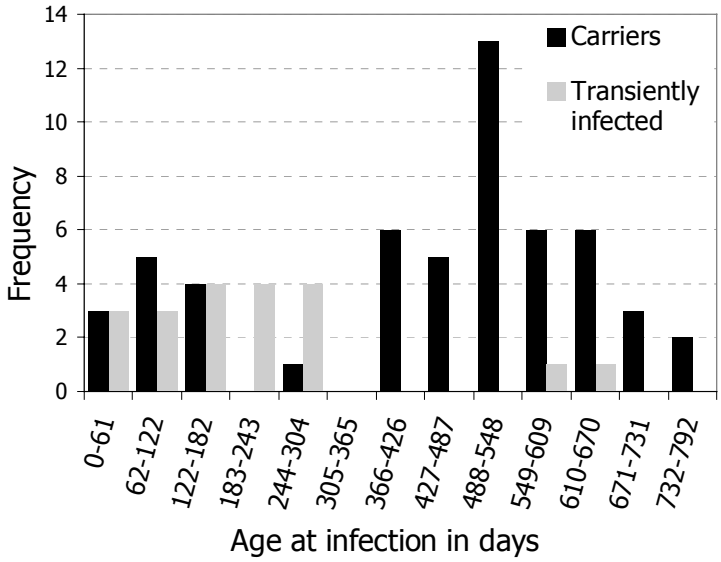


Fig. 4 Frequency distribution of *Salmonella* Dublin carriers and transiently infected animals among young calves and heifers in 12 dairy herds.

Discussion

Data quality

Concern about misclassification leading to biased results is always an issue in case-control studies. Some studies indicate that though long term carriers of *Salmonella* Dublin most commonly have long term antibody responses, a few may have a very vague immunological response (Gitter et al., 1978; Hoorfar et al., 1996). For this reason a small risk of misclassification between carrier animals and transiently infected is possible in the present study. Testing animals more frequently could have reduced this misclassification risk (i.e. sampling every month instead of every 3 months). Another possible source of bias is lack of sampling of certain age groups during the summer when young stock and heifers were kept out on pasture in some of the study herds. The model was tested with different data sets containing carrier animals and transiently infected animals categorized with both stricter and more relaxed criteria (e.g. categorized as transiently infected if test positive for less than 4 or 5 months and carriers if test positive for more than 6 or 7 months). Changing the definition for the categorization of infection courses would bring out such a selection bias, because with some criteria animals infected while out on pasture (not sampled) would be included in the model, and with other criteria they would not. The tested changes in the criteria for the categorization of animals as carriers and transiently infected did not have any

marked impact on the final model. Also apparent prevalence was tested with the model instead of the estimated true prevalence. This did not change the direction of the effect of the parameter estimates in the model. Apparent prevalence was also very significant when included in the model instead of true prevalence, and had a similar effect to true prevalence on the risk of becoming a carrier animal.

Age and time from calving

The results of this study showed a marked effect of age and lactation stage on the odds of becoming a carrier animal upon infection with *Salmonella* Dublin. Conditional on infection, heifers and cows around time of calving had significantly higher odds of becoming carriers than do cows in mid and late lactation. One other study suggested that septicemia in young susceptible calves is a likely mode of carrier production (House et al., 1993). The data in the present study are insufficient to support or rule out this finding. Calves in our study had almost equal odds of becoming carriers and becoming transiently infected. This may be due to differences in susceptibility between the calves. The importance of maternally derived salmonella specific antibodies on the infection course in young calves is unknown. In an experimental study of calves 50-80 days old, immunity from prior experimental *Salmonella* Dublin infection was shown to lower the concentration of bacteria in the gut lumen upon inoculation, but to increase the proportion of bacteria in lymph nodes of animals infected earlier (Steinbach et al., 1996). This may indicate that partly immune animals or animals exposed to low doses of *Salmonella* Dublin bacteria may have an increased risk of becoming carrier animals. Also, genetically determined resistance to *Salmonella* Dublin and intracellular organisms in general has been documented in cattle (Feng et al., 1996; Adams et al., 1999).

The significantly increased odds of becoming a carrier animal when infected close to calving support the hypothesis that stress is an important risk factor in the pathogenesis of development of a carrier type of infection. This is also supported by the fact that animals from herds that experienced disease outbreaks during the study period had higher overall odds of carrier development than animals from other herds in the study. The stress hypothesis is furthermore supported by a study in which dexamethasone injections in experimentally infected animals were used to induce long term mammary gland carriers of *Salmonella* Dublin (Spier et al., 1991).

Table 3 The resulting model using logistic regression analysis of risk factors for becoming a carrier as opposed to becoming transiently infected upon infection with Salmonella Dublin in 244 animals from 12 endemically infected Danish dairy herds in the years 2000-2002.

Risk factors	β	S.E.	P[a]	Odds ratio (OR)	95% CI of OR
Intercept	1.554	0.66			
Fixed effects					
Age & Time from calving			0.027		
Calves:	0.175	0.53		1.2	0.4 – 3.4
Heifers:	2.411	0.89		11.1	1.9 –63.8
Close to 1st calving:	1.396	0.64		4.0	1.2 – 14.1
Cows after 1st calving:	0.496	0.42		1.6	0.7 – 3.7
Close to 2nd or higher calving:	2.062	1.14		7.9	0.8 – 74.1
Cows after 2nd calving:	0.000	0.00		1.0	
Season			0.057		
1st quarter of the year	1.392	0.76		4.0	0.9 – 17.7
2nd quarter of the year	0.692	0.51		2.0	0.7 – 5.4
3rd quarter of the year	-0.408	0.63		0.7	0.2 – 2.3
4th quarter of the year	0.000	0.00		1.0	
Herd prevalence (TP[b])	-0.059	0.01	< 0.001	0.55[c]	0.5 – 0.6

Random effect	Variance component	S.E.	P[d]
Herd	1.0805	0.751	0.07

[a] P-value for each factor in the overall Type 3 statistic tests

[b] TP is the estimated true prevalence of *Salmonella* Dublin infected animals in the whole herd

[c] Odds ratio per 10% increase in herd prevalence

[d] Significance level of the variance component, herd, according to the Wald test statistics

Herd prevalence

Decreasing herd prevalence was a significant risk factor for carrier animal development upon infection. Assuming that the herd prevalence of potential shedders represents the level of exposure to *Salmonella* Dublin to the individual animal, it suggests that conditional on infection, animals exposed to lower doses of the infection have increased odds of becoming carriers as opposed to animals exposed to higher doses of infection. It does not, however, suggest that fewer carrier animals are being produced during times of high herd prevalence (i.e. during *Salmonella* Dublin related disease outbreaks), because overall, more animals are going to become infected during such times. The mechanism for the low level exposure

phenomenon is largely unknown, but is supported by the findings of Steinbach et al. (1996). It could be speculated that a certain threshold of infection dose is required for a sufficient cell-mediated immune response to eliminate the organism. If such a threshold (which may vary between animals due to differences in immunity and genetic resistance) is not reached, lack of immediate host response would allow the organism to access the lymphoid tissues and establish longterm, protected intracellular proliferation in macrophages.

Season

Season was found to be a significant predictor in the present study. Steffensen and Blom (1998) suggested that the seasonality often associated with *Salmonella* Dublin is due to above average temperatures during the Spring and Summer, which they found increased the incidence of clinical salmonellosis in the Fall. In this study there was a higher overall average of prevalence among all study herds in 2001 than in 2000, but since climate data were not collected, this theory can neither be confirmed nor refuted by this study. Another possible reason for the increased odds of becoming a carrier animal when infected in the first or second quarter of the year is the fact that the increased level of exposure during the Winter causes the level of immunity in the herd to be slightly higher when late Winter and Spring are reached. While increased level of immunity may cause fewer clinical cases to be seen in the herd, it may increase the risk of carrier production due to minimal immunological reactions in the individual animals upon infection.

Herd variation

Herd was included as a random effect in the logistic regression model. The interpretation of the random effect model is that animals in the study herds have different baseline odds of becoming carriers simply because they belong to different herds. Factors such as genetically determined resistance may influence the risk of becoming a carrier upon infection with *Salmonella* Dublin at animal level, but also at herd level. Differences in management, barn sectioning, herd size, feeding strategies and concurrent presence of other infections in the herd may also influence the capability of cattle to clear infection with *Salmonella* Dublin and thus contribute to the herd variation seen in the present study (Wray and Roeder, 1987; Losinger et al., 1997; Vaessen et al., 1998).

Model fit

The fit of the model was evaluated to be good according to residual plots and the deviance in relation to the degrees of freedom in the model. However, validation of the model with

new data is warranted. Another animal level factor that was not included in this model, but that may have an effect on the risk of becoming a carrier animal is the production level. In this study milk yield was not included in the model because almost half of the animals were young, non-yielding animals at time of infection. Data of milk yield from 82 cows in the study was collected (data not shown). There was no effect of being a high, low or average yielding cow on the odds of becoming a carrier upon infection (χ^2-test, p=0.92). Other factors that could have been included, if they had been available, to attempt to increase the model fit, were clinical registrations, vaccination history and antibiotic treatment around time of infection.

Conclusion

The present study examined factors influencing the risk of becoming a carrier upon infection with *Salmonella* Dublin as opposed to being transiently infected, using a case-control study of dairy cattle in 12 herds. The results showed that there were significantly higher odds of becoming carriers than transiently infected, if animals became infected as heifers (between 1 year of age and 1st calving) and cows around time of calving (±70 days from calving date) than if they became infected as cows during mid or late lactation. Other factors influencing whether animals became carriers upon infection included the time of the year and the level of exposure. Lower prevalence of potential shedders in the herd increased the odds of becoming carriers. Calves did not appear to have a higher risk of becoming carriers though they are usually the most commonly infected group of animals.

Acknowledgements

The authors wish to thank the farmers for participating in the project, the milk quality advisors of the Danish Dairy Board for collecting the samples and the technicians at The Veterinary Department, Steins Laboratory in Brørup, Denmark for handling and analyzing the samples with great dedication. The Kongeå-project of the Danish Dairy Board funded the project.

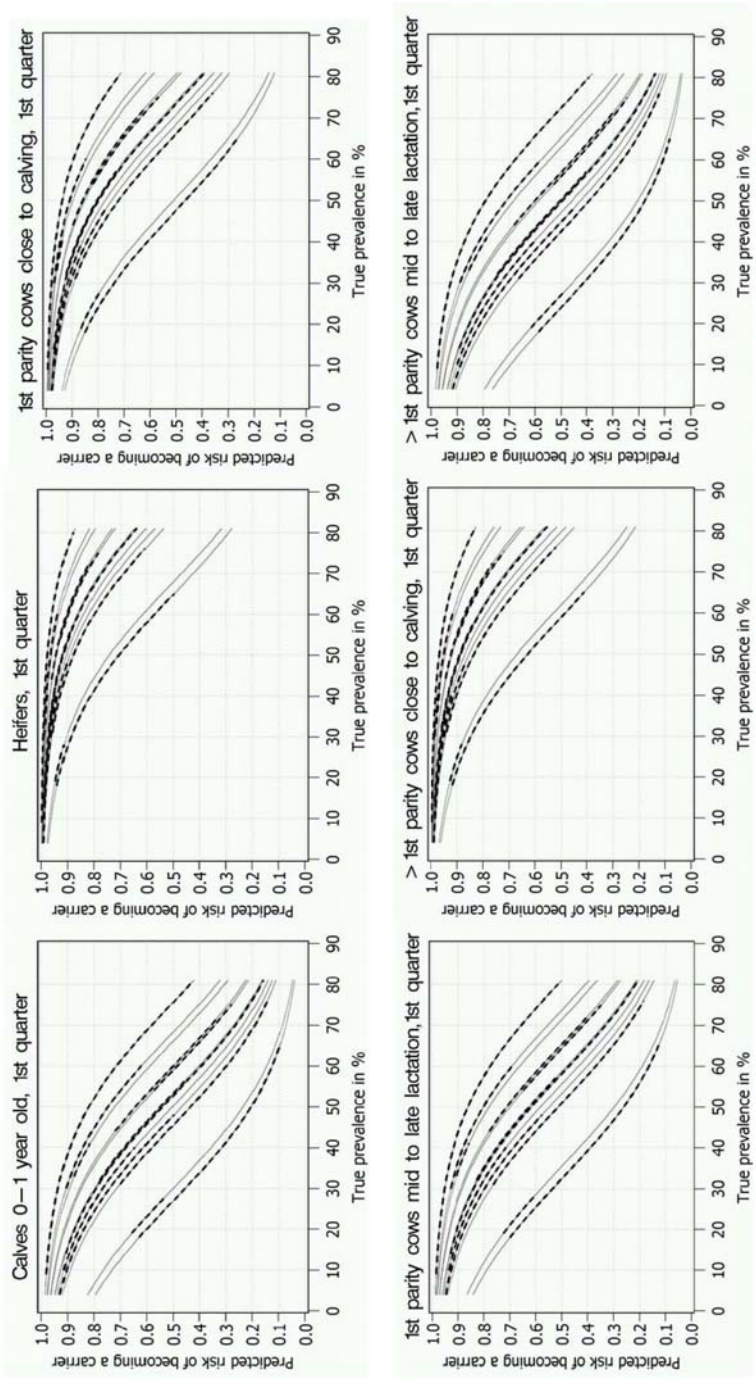


Fig. 5 Predicted risk of becoming a carrier upon infection with *Salmonella* Dublin plotted against estimated true prevalence of infected animals in the herd. The lines indicate the difference in predicted risks between the 12 herds included in the study. The predicted risks were calculated for 6 groups of animals in the season where infected animals had the highest risk of becoming carriers – i.e. January to March (1st quarter). For illustrative purposes the lines were extrapolated (gray lines) from the prevalence intervals actually found in the herds (dashed lines).

References

1. Adams, L.G., Barthel, R., Gutierrez, J.A. and Templeton, J.W., 1999. Bovine natural resistance macrophage protein 1 (NRAMP1) gene. Archiv Fuer Tierzucht 42, 42-54.

2. Andersen, H.J., Aagaard, K., Skjøth, F., Rattenborg, E. and Enevoldsen, C., 2000. Integration of research, development, health promotion, and milk quality assurance in the Danish Dairy Industry. *In*: Salman, M.D., Morley, P.S., Ruch-Galie, R. (Eds.), Proceedings of the 9th Symposium of the International Society of Veterinary Epidemiology and Economics., pp. 258-260.

3. Collett, D., 1991. Model checking. *In*: Modelling Binary Data, 1st Ed. Chapman & Hall, London, pp.

4. Counter, D.E., Gibson, E.A., 1980. Salmonella dublin infection in self contained dairy herds in East Anglia: excretion at calving. Vet. Rec. 107, 191-193.

5. Da Roden, L., Smith, B.P., Spier, S.J. and Dilling, G.W., 1992. Effect of calf age and Salmonella bacterin type on ability to produce immunoglobulins directed against Salmonella whole cells or lipopolysaccharide. Am. J. Vet. Res. 53, 1895-1899.

6. Feng, J., Li, Y., Hashad, M., Schurr, E., Gros, P., Adams, L.G. and Templeton, J.W., 1996. Bovine natural resistance associated macrophage protein 1 (Nramp1) gene. Genome Res. 6, 956-964.

7. Fierer, J., 1983. Invasive Salmonella dublin Infections Associated With Drinking Raw Milk. West. J. Med. 138, 665-669.

8. Gitter, M., Wray, C., Richardson, C. and Pepper, R.T., 1978. Chronic Salmonella dublin infection in calves. Br. Vet. J. 134, 113-121.

9. Gronstol, H., Osborne, A.D. and Pethiyagoda, S., 1974. Experimental Salmonella infection in calves. 1. The effect of stress factors on the carrier state. J. Hyg. 72, 155-162.

10. Hinton, M., 1974a. Isolation of Salmonella-Dublin from Fecal Swabs from Cattle. Br. Vet. J. 130, R31-R32.

11. Hinton, M., 1974b. Salmonella-Dublin Abortion in Cattle - Studies on Clinical Aspects of Condition. Br. Vet. J. 130, 556-563.

12. Hoorfar, J., Wedderkopp, A. and Lind, P., 1996. Comparison between persisting anti-lipopolysaccharide antibodies and culture at postmortem in salmonella-infected cattle herds. Vet. Microbiol. 50, 81-94.

13. House, J.K., Smith, B.P., Dilling, G.W., Da Roden, L., Spier, S.J. and Picanso, J., 1991. Serologic detection of Salmonella dublin infection in cattle. *In*: Proceedings of the Ninth Annual Veterinary Medical Forum., pp. 563-566.

14. House, J.K., Smith, B.P., Dilling, G.W. and Roden, L.D., 1993. Enzyme-linked immunosorbent assay for serologic detection of *Salmonella dublin* carriers on a large dairy. Am. J. Vet. Res. 54, 1391-1399.

15. Humphrey, T., Wray, C. and Wray, A., 2000. Public-health aspects of Salmonella infection. *In*: Wray, C., Wray, A. (Eds.), Salmonella in Domestic Animals. CABI Publishing, New York, New York State, pp. 245-263.

16. Losinger, W.C., Garber, L.P., Smith, M.A., Hurd, H.S., Biehl, L.G., Fedorka-Cray, P.J., Thomas, L.A. and Ferris, K., 1997. Management and nutritional factors associated with the detection of Salmonella sp. from cattle fecal specimens from feedlot operations in the United States. Prev. Vet. Med. 31, 231-244.

17. Martin, S.W., Meek, A.H. and Willeberg, P., 1987. Veterinary Epidemiology, 1st Ed. Iowa State University Press, Ames, Iowa.

18. Morisse, J.P., Cotte, J.P., 1994. Evaluation of some risks factors in bovine salmonellosis. Vet. Res. 25, 185-191.

19. Nielsen, L.R., Ersbøll, A.K., In press. Age stratified validation of an indirect *Salmonella* Dublin serum ELISA for individual diagnosis in cattle. Journal of Veterinary Investigation.

20. Peters, A.R., 1985. An Estimation of the Economic-Impact of An Outbreak of Salmonella-Dublin in A Calf Rearing Unit. Vet. Rec. 117, 667-668.

21. Richardson, A., Fawcett, A.R., 1973. Salmonella-Dublin Infection in Calves - Value of Rectal Swabs in Diagnosis and Epidemiological Studies. Br. Vet. J. 129, 151-156.

22. Robertsson, J.A., 1984. Humoral antibody responses to experimental and spontaneous Salmonella infections in cattle measured by ELISA. Zentralbl. Veterinarmed. B. 31, 367-380.

23. Schønheyder, H.C., Kristensen, L., Lester, A., Scheibel, J.H. and Gerner-Smidt, P., 1997. Ekstraintestinale Salmonella-infektioner i fire danske amter. Ugeskr Læger 159, 2082-2085.

24. Smith, B.P., House, J.K., Dilling, G.W., Roden, L.D. and Spier, S.J., 1992. Identification of Salmonella Dublin Carrier Cattle. *In*: Proceedings of the International symposium Salmonella and salmonellosis. Zoopôle, Ploufragan, France., pp. 225-230.

25. Smith, B.P., Oliver, D.G., Singh, P., Dilling, G., Marvin, P.A., Ram, B.P., Jang, L.S., Sharkov, N. and Orsborn, J.S., 1989. Detection of Salmonella dublin mammary gland infection in carrier cows, using an ELISA for antibody in milk or serum. Am. J. Vet. Res. 50, 1352-1360.

26. Smith, R.D., 1995. Veterinary Clinical Epidemiology. A Problem-Oriented Approach, 2 Ed. CRC Press, Inc., Boca Raton.

27. Spier, S.J., Smith, B.P., Cullor, J.S., Olander, H.J., Da Roden, L. and Dilling, G.W., 1991. Persistent Experimental Salmonella dublin Intramammary Infection in Dairy Cows. J. Vet. Int. Med. 5, 341-350.

28. Spier, S.J., Smith, B.P., Tyler, J.W., Cullor, J.S., Dilling, G.W. and Da Pfaff, L., 1990. Use of ELISA for detection of immunoglobulins G and M that recognize Salmonella dublin lipopolysaccharide for prediction of carrier status in cattle. Am. J. Vet. Res. 51, 1900-1904.

29. Steffensen, M., Blom, J.Y., 1999. Forekomsten af salmonella-infektioner i danske kvaegbe-saetninger 1992-1998. (Incidence of Salmonella infections in Danish cattle herds 1992-1998). Dan. Veterinærtidsskr. 82, 966-970.

30. Steinbach, G., Koch, H., Meyer, H. and Klaus, C., 1996. Influence of prior infection on the dynamics of bacterial counts in calves experimentally infected with Salmonella dublin. Vet. Microbiol. 48, 199-206.

31. Vaessen, M.A., Veling, J., Frankena, K., Graat, E.A. and Klunder, T., 1998. Risk Factors for *Salmonella Dublin* infection on Dairy Farms. Vet. Quart. 20, 97-99.

32. Veling, J., van Zijderveld, F.G., Zijderveld-van Bemmel, A.M., Barkema, H.W. and Schukken, Y.H., 2000. Evaluation of three newly developed enzyme-linked immu-nosorbent assays and two agglutination tests for detecting Salmonella enterica subsp enterica serovar Dublin infections in dairy cattle. J Clin. Microbiol. 38, 4402-4407.

33. Visser, S.C., Veling, J., Dijkhuizen, A.A. and Huirne, R.B.M., 1997. Economic losses due to Salmonella dublin in dairy cattle. *In*: Proceedings of the Dutch/Danish Symposium on Animal Health and Management Economics, Copenhagen., pp. 143-151.

34. Wedderkopp, A., Stroger, U., Bitsch, V. and Lind, P., 2001. Testing of bulk tank milk for Salmonella Dublin infection in Danish dairy herds. Can. J. Vet. Res. 65, 15-21.

35. Wray, C., Davies, R.H., 2000. Salmonella infections in cattle. *In*: Wray, C., Wray, A. (Eds.), Salmonella in Domestic Animals. CABI Publishing, New York, New York State, pp. 169-190.

36. Wray, C., Roeder, P.L., 1987. Effect of bovine virus diarrhoea-mucosal disease virus infection on salmonella infection in calves. Res. Vet. Sci. 42, 213-218.

37. Wray, C., Snoyenbos, G.H., 1985. Salmonella dublin infection of cattle in England and Wales: its epidemiology and control. *In*: Snoyenbos, G.H. (Ed.), Proceedings of the International Symposium on Salmonella, New Orleans., pp. 173-181.

38. Wray, C., Todd, N., McLaren, I.M. and Beedell, Y.E., 1991. The epidemiology of salmonella in calves: the role of markets and vehicles. Epid. Infect. 107, 521-525.

Appendix A: Contents of CASADY-datasets

"CASADY1"

SAS-dataset (Version 8.2) containing bulk tank milk ELISA results

Variables and coding

Loebenr	Unique running number for each sample
Chrnr	Herd number from the Danish Cattle Database
Dato	Date of sample collection
Regdato	Date of registration
DubODCp	ELISA response against *Salmonella* Dublin in bulk tank milk sample
TyphODCp	ELISA response against *Salmonella* Dublin in bulk tank milk sample
Pladenr	Platenumber from the ELISA-test
Proevenr	Testnumber (position in the ELISA plate)

"CASADY2", "CASADY3" and "CASADY4"

(SAS-data sets (Version 8.2) containing ELISA and faecal culture results from Act#2, Act#3 and Act#4 – with animal characteristics from the Danish Cattle Database.

Variables and coding

Loebenr	Unique sample number (from DHC-Lab-Filemaker-files)
Chrnr	Herd identification number in Danish Cattle Database
Ckrnr	Individual identification number of animals
Kirtel	For milk samples: Indication of which quarter the sample is from
	1: Right front (HF)
	2: Left front (VF)
	3: Right rear (HB)
	4: Left rear (VB)
	5: Mix from several or all quarters of the udder
Sampdate	Date sample was collected in herd
BC	Bacterial culture result
	0: 0: Negative culture result
	1: Dublin
	2: Typhimurium
	3: Rough salmonella type
	4: Other salmonelle types (e.g. Saintpaul, Agona, Derby)
herdstat	Based on all culture results from the respective herd
	0: No salmonella bacteria found
	1: Only Salmonella Dublin found
	2: Only Salmonella Typhimurium found
	3: Mainly Salmonella Dublin, but also other serotypes found
	4: Uncertain status (sporadic finding of 1 other salmonella serotype or very high serology, but no bacterias found)
born	Date of birth
Indate	Date of entrance into herd
Outdate	Date of departure from herd
Outcause	Reason for leaving herd:
	7: Slaughter
	9: Dead
	16: Sold
	50: Out-stationed in other herd
breed	
	1: Red Danish (RDM)
	2: Holstein-Friesean (SDM)
	3: Jersey
	8: Crosses and other breeds (DRK, Simmental, etc.)
materckr	Individual identification number of mother to tested animal
alder	Age in days on days sample was collected
DubODCPm	ELISA response (ODC%) in Dublin-milk ELISA
TyphODCPm	ELISA response (ODC%) in Typhimurium-milk ELISA
DubODCPs	ELISA response (ODC%) in Dublin-serum ELISA
TyphODCPs	ELISA response (ODC%) in Typhimurium-serum ELISA

"CASADY5"

SAS-dataset (Version 8.2) containing register-data of individual animals from the Danish Cattle Database.

Variables and coding

CHRNRK	Herd ID-number (5 digits)
CKRNR	Animal ID-number (9 digits)
BORN	Date of birth
INDATE	Date of entrance to herd (usually the same as date of birth if born in herd)
OUTDATE	Date of departure from herd
OUTCAUSE	(According to coding from the Danish Cattle Database)
	7: Slaughter
	9: Dead
	16: Sold
	50: Out-stationed in other herd
DYRESTAT	(According to coding from the Danish Cattle Database)
	1: Bull
	2: Heifer
	9: Cow
BREED	(Modified from the Danish Cattle Database)
	1: Red Danish (RDM)
	2: Holstein-Friesean (SDM)
	3: Jersey
	8: Crosses and other breeds (DRK, Simmental, etc.)
MATERCKR	Mother's ID-number
KLVDATO	Calving date
PARITET	Parity relating to registered calving date
KTRDATO	Milk control date (date of registry of calving date, parity, milk yield etc.)
KGMLK	Daily milk yield on control date
FEDPCT	Fat percent in milk on control date
PROTEINPCT	Protein percent in milk on control date
CELLETAL	Somatic cell count in milk on control date

Appendix B Overview of herds participating in sampling activities number 2, 3 and 4.

Herd number	Bulk milk ELISA response	Vet practice	Type prod. system	Postal zip code	Number of cows	Barn type	Breed
1	Dublin high	814	Trad.	6630	90	Loose housing	SDM
2	Dublin high	833	Trad.	6520	51	Tie stalls	SDM
3	Typhimurium high	814	Trad.	6630	43	Loose housing	SDM
4	Dublin high	814	Trad.	6630	51	Tie stalls	SDM
5	Dublin high	833	Trad.	6520	70	Tie stalls	Jersey
6	Dublin high	1017	Trad.	6630	58	Tie stalls	SDM
7	Dublin high	816	Trad.	6630	104	Loose housing	SDM
8	Dublin high	916	Trad.	6660	80	Tie stalls	SDM
9	Dublin high	838	Trad.	6520	117	Loose housing	SDM
10	Dublin high	916	Trad.	6630	43	Loose housing	Jersey
11	Dublin high/mid	833	Trad.	6520	57	Tie stalls	SDM
12	Dublin high	838	Trad.	6520	67	Tie stalls	Jersey
13	Dublin high/mid	816	Organic	6520	75	Loose housing	SDM
14	Typhimurium outbreak	928	Trad.	6630	91	Tie stalls	SDM
15	Dublin high/mid	804	Trad.	6630	76	Tie stalls	SDM
16	Dublin mid	816	Trad.	6510	70	Tie stalls	SDM
17	Dublin mid	814	Trad.	6630	62	Tie stalls	SDM
18	Dublin low	814	Trad.	6630	61	Loose housing	SDM
19	Dublin high	833	Trad.	6520	71	Tie stalls	SDM
20	Dublin high/mid	838	Organic	6520	107	Loose housing	SDM

(Appendix B continued)

Herd number	Bulk milk ELISA response	Vet practice	Type prod. system	Postal zip code	Number of cows	Barn type	Breed
21	Dublin mid	814	Organic	6630	95	Loose housing	SDM
22	Dublin low	847	Trad.	6630	52	Tie stalls	SDM
23	Dublin high/mid	916	Trad.	6660	73	Tie stalls	Jersey
24	Dublin mid	814	Trad.	6630	78	Loose housing	SDM
25	Dublin mid	814	Trad.	6510	80	Loose housing	SDM
26	Dublin low	847	Trad.	6630	58	Tie stalls	SDM
27	Dublin mid	814	Trad.	6660	31	Tie stalls	SDM
28	Dublin mid	814	Trad.	6630	90	Tie stalls	SDM
29	Dublin low	816	Trad.	6510	60	Tie stalls	SDM
30	Dublin outbreak	816	Trad.	6510	130	Loose housing	SDM
31	Salmonella outbreak suspected	847	Trad.	6630	55	Tie stalls	SDM
32	Salmonella outbreak suspected	838	Trad.	6520	100	Tie stalls	SDM
33	Salmonella outbreak suspected	816	Trad.	6510	130	Loose housing	SDM
34	Salmonella outbreak suspected	838	Trad.	6520	100	Loose housing	SDM
35	Salmonella outbreak suspected	814	Trad.	6630	90	Loose housing	SDM