



**Paratuberculosis in Danish dairy cattle
interpretation of diagnostic information depending on purpose and disease stage**

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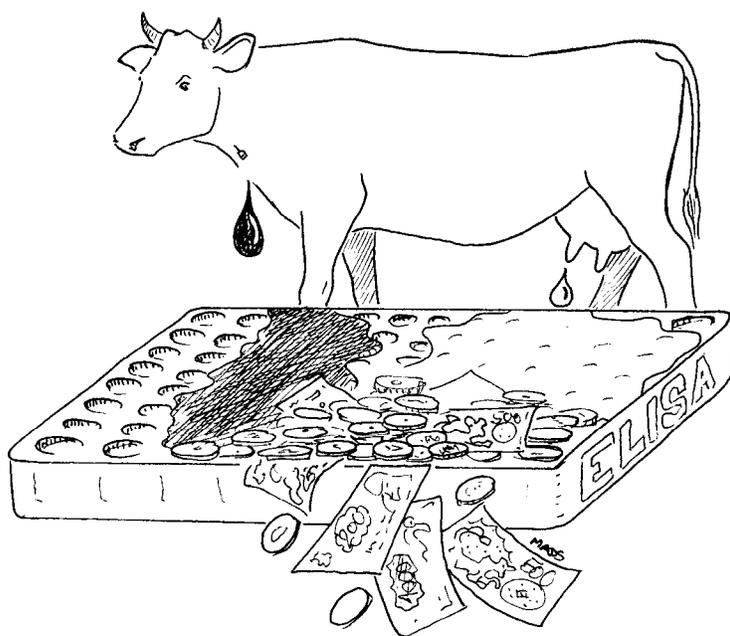
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Søren Saxmose Nielsen

Paratuberculosis in Danish dairy cattle

- Interpretation of diagnostic information depending on purpose and disease stage



Ph.D. Thesis

Epidemiology

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The Royal Veterinary and Agricultural University

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Paratuberculosis in Danish dairy cattle

- Interpretation of diagnostic information depending on purpose and disease stage

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Preface

‘Kongeåprojektet’ (the Kongeå-project) - aimed at establishing a cattle-industry-based integrated research-project on veterinary and milk quality issues - was initiated in 1997. The project was established under the responsibility of Karsten Aagaard, The Veterinary and Milk Quality Department, Danish Dairy Board, with financial contributions from the dairy farmers and livestock production. Meanwhile, at the Cattle Health Laboratory (CHL) in Ladelund, a fellow was attending ELISA-plates, attempting to fill small wells with various liquids for known as well as for unknown purposes.

In 1999 the Kongeå-project hired the first of a total of nine ph.d. students for the project – with partial funding from the Danish Research Academy. Meanwhile, at the CHL, one Viggo Bitsch tried to shape the fellow to do something beside filling and emptying the ELISA wells. The result thereof was enrolment of the fellow in a ph.d. project on the epidemiology of paratuberculosis. Ph.d. project planning commenced, supervised by Jens Frederik Agger and Peter Lind in collaboration with Anna Huda, Anna Bodil Christoffersen, Anne Kudahl, Bent Aalbæk, Carsten Enevoldsen, Kirstine Klitgaard Nielsen, Peter Ahrens, Steen Giese and Viggo Bitsch. The plans were carried out, samples were collected by practising veterinarians and the milk quality advisors Bent Jensen, Bent Truelsen, Jan Nelson, Lars Mortensen, Niels Sørensen. The samples were administrated primarily by Anne-Marie Sørensen assisted by Sanne Prüsse and Heidi Jørgensen at CHL. Anne-Marie skilfully made sure that the samples were registered so they could be found again. The collected milk and serum samples were analysed by Laila Rossen and Ulla Østerby, with prior handling assisted by most employees at CHL and supervised by Viggo Bitsch. The collected faecal samples were analysed by Ingerlise Christensen, Inga Filtenborg, Kirsten Bak, Sonja Kjær, Tina Iversen, Randi Carlsen and Rikke Jensen supervised by Anna-Bodil Christoffersen. Meanwhile, the fellow was fooling around in places where he would destroy as few things as possible for others. Among other things the fellow was enrolled in the Research School for Animal Production (RAPH) and participated in the fabulous activities of this research school joyfully lead by Pia Haubro Andersen. The fellow also went to, Cornell University, New York, where Yrjö Gröhn tried training the

fellow in marathoning, both the running-in-the-street version as well as marathoning with data. Ynte Schukken, Gerdien van Schaik, Richard Jacobson, Christine Rossiter and Susan Stehman (all Cornell University) contributed in the discussions on the data analysis. Julia Hertl (also from Cornell University) proofread this manuscript. Persistent Trans-Atlantic communication with Carsten Enevoldsen during the data marathoning also constituted a significant part of the data analysis. Parts of these data were retrieved and administrated from the Danish Cattle Database by Marianne Skovbogaard, who also edited whatever rubbish the fellow wrote into readable stuff for the newsletter "Kongeåprojektet". Following the stay at Cornell, the fellow stayed home at the Department of Animal Science and Animal Health, KVL where the staff helped making everyday life pleasant. Once in a while he still aimlessly filled ELISA-wells, but most of the time the fellow bartered Liza Nielsen, Hans Houe and Nils Toft with tall stories from real life while they tried to ask questions that could relate the fellow's paratuberculosis-stuff to real life. When the fellow did not tell tall stories and did not purposelessly fill ELISA-wells, he wrote the stuff you have in your hands. Stine Jacobsen, who repeatedly uttered that he was the best ph.d.-student she knew, stimulated the process significantly by saying so even though she had no clue what he was doing in spite the fact that all the other people mentioned in the above actually did most of the job. The siblings of the fellow, Malene, Jakob and Peter, and the sire and dam of all these persons also stimulated the process of the present work.

The fellow wishes to thank all of the mentioned persons for doing all the stuff mentioned, including all the help provided that is not mentioned. The fellow would also like to express his gratitude toward the involved organisations for funding the projects and thereby providing the basis of the study. The front page of this thesis may indicate that the fellow takes the entire credit for the thesis. However, it is really the result of the joint effort of all these persons. In reality they are the ones that should really be acknowledged.

Frederiksberg, May 2002

Søren Saxmose Nielsen

List of papers

- Paper I SS Nielsen, H Houe, SM Thamsborg, V Bitsch (2001)
Comparison of two enzyme-linked immunosorbent assays for serological diagnosis of paratuberculosis (Johne's disease) in cattle using different subspecies strains of *Mycobacterium avium*. *Journal of Veterinary Diagnostic Investigation*, **13**, 164-166.
- Paper II SS Nielsen, C Grøn­bæk, JF Agger, H Houe (2002)
Maximum-likelihood estimation of sensitivity and specificity of ELISAs and faecal culture for diagnosis of paratuberculosis. *Preventive Veterinary Medicine*, **53**, 189-202.
- Paper III SS Nielsen
Variance components of an enzyme-linked immunosorbent assay for detection of IgG antibodies in milk samples to *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. Submitted for publication in *Journal of Veterinary Medicine, series B*.
- Paper IV SS Nielsen, C Enevoldsen, YT Gröhn (2002)
The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. *Preventive Veterinary Medicine*, **54**, 1-10.
- Paper V SS Nielsen, YT Gröhn, C Enevoldsen
Variation of the milk antibody response to paratuberculosis in naturally infected dairy cows. Submitted for publication in *Journal of Dairy Science*.

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Summary

Mycobacterium avium subsp. *paratuberculosis* is prevalent in the Danish dairy population. Presence of the organism can lead to clinical disease, which may result in production loss. The prevalence of paratuberculosis as a clinical entity is not known, nor is the prevalence of animals transmitting the infective agent to other cows. These might be among the most interesting prevalences, but paratuberculosis can be defined in many ways. However, ultimately the purpose of reporting should assume a definition of paratuberculosis that considered one of the following purposes: i) individual animal welfare; ii) production loss; and iii) transmission of infection. Other purposes would be assumed to be derivatives thereof.

The laboratory tests used to detect *M. avium* subsp. *paratuberculosis* and infections with the bacteria generally suffer from a low sensitivity and specificity. Lack of sensitivity is primarily due to the chronic nature of paratuberculosis and possible latency of the infection. Lack of specificity is due to the widespread occurrence of bacteria with features very similar to *M. avium* subsp. *paratuberculosis*, including the immunogenicity of these bacteria. Correct quantification of the sensitivity and specificity are essential in calculations of the possibilities of predicting the efforts necessary to initiate an action against *M. avium* subsp. *paratuberculosis*. Subsequently, the diagnostic tests have to be cost-effective to serve their purpose. The sensitivity and specificity change with the stage of infection. Thus, purpose-related and disease stage interpretation of the diagnostic tests are necessary.

The objective of this thesis is to give an epidemiological evaluation of the usefulness of the diagnostic information obtained from laboratories and to use the information to study the dynamics of the disease with description of cow types that can be applied in decision support systems and by farmers in control of the infection.

Chapter 1 provides a general introduction into the history and historical definitions of paratuberculosis. A literature review of the occurrence of paratuberculosis, aetiology and immunology, pathogenesis and diagnostic possibilities

is given along with a more elaborate description of the hypothesis and objectives of the thesis.

Chapter 2 describes evaluation of the diagnostic tests used in the thesis, namely enzyme-linked immunosorbent assays (ELISA) for detection of antibodies in milk and serum, and detection of *M. avium* subsp. *paratuberculosis* in faecal samples (faecal culture). First, an example of biased sampling, which would lead to overestimation of the sensitivity of the ELISA, is provided. This study also aims to determine the relative specificity of the antigen used in the ELISA. The antigen is from *M. avium* subsp. *avium*, but apparently it is as specific as a *M. avium* subsp. *paratuberculosis* antigen. Second, a study to determine the sensitivity and specificity of both ELISA and faecal culture is described based on cows from all likely stages of infection, including latent stages of disease. The linkage between sensitivity and specificity of ELISA and faecal culture is described and forms the basis for drawing attention to two cut-off points of the ELISA. One is where antibody level is high. High antibody level results in a high sensitivity of faecal culture. Low antibody level results in low sensitivity of faecal culture. The approach demonstrates the significance of choice of definition of paratuberculosis on the performance of the tests used.

In a separate study, the variability of the ELISA is demonstrated and important variation points are demonstrated. Significant variation is recorded between ELISA-plates and test-days. Thus, this information needs to be considered in further statistical analyses of the data described.

In Chapter 3, the cow-time factors of importance in the ELISA interpretation are described. The ELISA results are more likely to be positive in 2nd and higher parities relative to 1st parity. For milk ELISA, results obtained from cows in mid-lactation are less likely to be positive than results obtained from cows at the beginning and the end of the lactation. For serum ELISA, an almost inverted situation is present. These effects are studied further in cows from 7 herds known to be infected with paratuberculosis. Milk was collected continuously from these cows and the dynamics of the ELISA readings described for both cows with positive and negative faecal culture. Cow profiles were demonstrated and their shapes were not related to the faecal

culture group but to some other factors. The sensitivity of the ELISA could be increased 8% with repeated sampling.

In Chapter 4, the results presented in the previous chapters are linked together and syntheses made including critical remarks and potential drawback of the studies the linkages. Theoretical static and dynamic cows are described based on the findings in Chapters 2 and 3. Four types are identified: a) Not infected; b) Infected, 'controlling the infection' ('Inactive ParaTB'); c) Infected, initially 'controlling' the disease but loosing control at a sudden point in time ('Active ParaTB'); and d) Infected, not 'controlling the infection' ('Active ParaTB'). The cow types are suggested to form the basis for cow definitions in the future control of paratuberculosis. These cow types can be included in a testing scheme, which also includes defining purpose prior to the testing.

In Chapter 5, the main results are listed, and concluding remarks and perspectives are made.

Sammenfatning (Summary in Danish)

Forekomsten af *Mycobacterium avium* subsp. *paratuberculosis* er hyppig i den danske malkekvægspopulation. Tilstedeværelsen af organismen kan medføre klinisk sygdom, herunder produktionstab. Prævalensen af klinisk paratuberkulose er ukendt ligesom forekomsten af smitteudskillende dyr ikke kendes med sikkerhed. Disse prævalenser er nok blandt de mest interessante, men paratuberkulose kan defineres på mange måder. I sidste ende er det dog normalt formålet med en given rapportering, der bør bestemme hvorledes man definerer paratuberkulose. Derfor vil de hyppigst anvendte formål nok falde indenfor en af følgende: i) velfærd for det enkelte dyr; ii) produktionstab; iii) spredning af infektionen. Andre formål må betragtes som derivater af disse.

De laboratorietest, der normalt anvendes til påvisning af *M. avium* subsp. *paratuberculosis* og infektion med samme, har generelt lav sensitivitet og specificitet. Den manglende sensitivitet hænger primært sammen med sygdommens kroniske natur og forekomsten af latente infektioner. Manglende specificitet kan skyldes forekomsten af bakterier, der er nært beslægtet med *M. avium* subsp. *paratuberculosis*, og som derfor har mange fælles træk med denne, herunder en immunogenicitet, der ligner en del. Korrekt kvantificering af sensitivitet og specificitet er nødvendig for at bestemme de prædiktive evner af de anvendte test, når disse bruges i forskellige tiltag mod *M. avium* subsp. *paratuberculosis*. Desuden skal prisen på de anvendte test stå mål med effektiviteten af dem. Da sensitiviteten og specificiteten af de anvendte test ændres med sygdommens forskellige stadier, er formålsrettet og sygdomsstadie relateret tolkning af de diagnostiske test nødvendig.

Formålet med denne afhandling er at give en epidemiologisk vurdering af anvendeligheden af den diagnostiske information, der kan indhentes fra laboratorietest og anvende denne information til at studere dynamikken af sygdommen med henblik på en beskrivelse af ko-typer, som kan anvendes i beslutningsstøttesystemer og af landmænd, når infektionen skal bringes under kontrol.

Kapitel 1 giver en generel introduktion til sygdommens historie, herunder historiske definitioner af paratuberkulose. På basis af litteraturstudier opremses forhold

vedrørende forekomst, ætiologi, immunologi, patogenese og diagnostiske muligheder, mundende ud i en mere dybdegående beskrivelse af hypotese og formål med afhandlingen.

Kapitel 2 beskriver evaluering af de diagnostiske test, der anvendes i studier i afhandlingen, nemlig enzyme-linked immunosorbent assay (ELISA) til bestemmelse af antistoffer i mælk og blod, og påvisning af *M. avium* subsp. *paratuberculosis* i gødningsprøver (bakteriologisk dyrkning). Indledningsvist bringes et eksempel på, hvorledes man kan opnå overestimering af sensitiviteten af ELISA ved ikke at udvælge sine studie-objekter tilfældigt. Dette studie har også til formål at bestemme den relative specificitet af ELISA. Det anvendte antigen stammer fra *M. avium* subsp. *avium*, men det er tilsyneladende lige så specifikt som hvis et *M. avium* subsp. *paratuberculosis* antigen havde været anvendt. Efterfølgende blev der udført et studie, der havde til formål at bestemme sensitivitet og specificitet af både ELISA og bakteriologisk dyrkning hos køer fra alle stadier af paratuberkulose-infektion, inklusive dyr, der er i det latente sygdomsstadium. Koblingen mellem sensitivitet og specificitet mellem ELISA og bakteriologisk dyrkning beskrives og danner basis for anvendelse af to cut-off værdier i ELISAen. Den ene cut-off værdi er, hvor antistof-niveauet er højt, og høje antistof-niveauer resulterer i høj sensitivitet af den bakteriologiske dyrkning. Lave antistof-niveauer resulterer i lav sensitivitet af den bakteriologiske dyrkning. Fremgangsmåden demonstrerer betydningen af, hvilken definition af paratuberkulose man lægger til grund for evalueringen af testen.

I et separat studium bestemmes bidraget fra forskellige komponenter af laboratorie-variation for ELISAen. Der er signifikant variation mellem ELISA-plader og test-dage. Derfor bør denne information inkluderes, når der foretages statistiske analyser af data, der oprinder fra ELISA-analyser.

I kapitel 3 beskrives ko-tids-faktorer, der er vigtige i tolkningen af ELISA. Sandsynligheden for at være positiv i ELISA er mindst for 1. kalvskøer i forhold til øvrige køer. Mælke-ELISA resultater fra prøver udtaget af køer midt i laktationen er mindre sandsynlige at være positive end prøver udtager fra køer først eller sidst i laktationen. Serum-ELISA resultater viser et billede, der er næsten omvendt. Nogle af

disse effekter studeres nærmere hos køer fra 7 besætninger, der alle vides at have *M. avium* subsp. *paratuberculosis* i besætningen. Mælkeprøver indsamles gentagne gange fra disse køer, og dynamikken af ELISA-resultaterne beskrives for køer, for hvilken bakteriologisk dyrkning også er kendt. Køernes antistof-profiler beskrives, og deres form er ikke relateret til dyrknings-gruppe, men til andre faktorer. Sensitiviteten af ELISA stiger med 8% ved gentagen prøveindsamling.

I kapitel 4 sammenkædes resultaterne fra de forrige kapitler og uddybende kritiske bemærkninger til sammenkædningen angives, inkluderende en diskussion af de potentielle bias som sammenfatning kunne føre til. Der beskrives statiske og dynamiske antistof-profiler for køer baseret på fundene i kapitlerne 2 og 3. Fire ko-typer beskrives: a) Ikke-inficeret; b) Inficeret med 'kontrol' over infektionen ('Inaktiv ParaTB'); c) Inficeret, indledningsvist med 'kontrol' over infektionen, men hvor denne kontrol mistes på et givent tidspunkt ('Aktiv ParaTB'); og d) Inficeret, uden 'kontrol' med infektionen ('Aktiv ParaTB'). Ko-typerne foreslås at danne basis for definition af ko-typer i et fremtidigt paratuberkulose kontrolprogram i Danmark. Ko-typerne kan inkluderes i et testnings-system, som også skal inkludere en definition af formålet med at teste en ko.

I kapitel 5 præsenteres hovedresultater og konklusioner og perspektiver oplistes.

Chapter 1. General introduction

Introduction

‘Paratuberculosis is a chronic granulomatous gastrointestinal disorder of ruminants. The causative agent is *Mycobacterium avium* subsp. *paratuberculosis*’. This phrasing could be a general introduction to a scientific report on paratuberculosis and the definition would gain general acceptance from most pathologists and clinicians. However, the applicability of this definition from a disease prevention point of view is in some terms unfortunate. What is paratuberculosis? – and how should it be defined?

History and historical definition

In the 20th century, the main part of the history of paratuberculosis was formed and described (Chiodini, 1993). The disease entity gained the nowadays most common names Johne’s disease (named by McFadyen (1906)) and paratuberculosis (named by Bang (1906)). The disease gained these names following the pathological description (Johne and Frothingham, 1895) and the induction of experimental disease in calves fed infected intestine from infected cows (Bang, 1906). These descriptions have set the standards of what is considered paratuberculosis at the entry to the 21st century. However, as early as 1807, a disease entity called “Consumption” or “Wasting” (Skellet, 1811) with some clinical features similar to that of “clinical paratuberculosis”¹ was described. And subsequently, a number of descriptions from clinical practice were reported in the first half of the 19th century (Cartwright, 1829; 1831). None of these cases will probably ever be recorded as “true paratuberculosis cases”. Were they the result of infection with *M. avium* subsp. *paratuberculosis*? And should they have been classified as paratuberculosis when the diagnosis could not be confirmed? The veterinarian in the 19th century would have to base his diagnosis and

¹ “Clinical disease” here used according to Whitlock and Buergelt (1996): “... gradual weight loss despite a normal or, occasionally, an increased appetite. During a period of several weeks, concurrent with the weight loss, the manure consistency becomes more fluid. The diarrhea may be intermittent at first, with periods of normal manure consistency.....As the disease progresses, the affected animals become increasingly lethargic, weak, and emaciated.....”

treatment on the clinical findings because the disease was not defined. Since Johne and Frothingham described the disease and Bang induced it experimentally, the definitive diagnosis could potentially be established. However, a definitive diagnosis can only be established in some cases most often as a definitive positive diagnosis when pathological lesions and the bacteria have been identified. Because paratuberculosis can appear as a latent infection (Whitlock and Buergelt, 1996) some cows will be designated “non-paratuberculosis” by use of a definitive diagnostic system. Rather than a diagnosis based on clinical findings or a diagnosis based on a definitive test, a system that can comprise more types of paratuberculosis would seem desirable. This system could incorporate a probability diagnosis. Normally, the latent infections cannot be detected with 100% certainty by use of the available tests. Therefore, the use of a probability diagnosis would usually be the outcome when testing these animals. However, a characterisation of the dynamics of the infection in naturally infected cows would be necessary to provide a probable probability diagnosis. And definitions that concur with the events in the diagnostic tests are therefore desirable. To date, these definitions have comprised terms like “silent”, “latent”, “subclinical”, “clinical”, “with pathological findings consistent with paratuberculosis” etc. Such terms are not necessarily concordant with the desires of a control program to prevent future disease. A set of definitions where the diagnostic tests and the legal measures are in agreement would therefore be necessary in preventive veterinary medicine.

Occurrence and significance

Paratuberculosis is fairly prevalent in most Western European countries with reported prevalences up to more than 50% of the herds in The Netherlands and a few percent of the individuals in various countries (Kennedy and Benedictus, 2001). However, different methods of estimating the prevalence in different countries make inter-country comparison difficult.

The prevalence and incidence of paratuberculosis in the Danish cattle industry is not known with certainty. To estimate the prevalence, a strict definition of ‘paratuberculosis’ is needed and a precise test to detect these animals is also needed.

Any prevalence estimate will depend on the acceptance of a number of assumptions most of which are related to the chronicity of the disease. Because neither a strict definition nor a perfect test exists, true prevalence estimates are therefore difficult to obtain. Estimates of the incidence are even more difficult to obtain because of lack of knowledge of the incubation period. Usually, infection is assumed to take place in early calthood, but this assumption is also difficult to validate.

In 1998, a study was carried out to estimate the herd-level prevalence in Denmark. In total, 48% of the herds included in the study had evidence of paratuberculosis infection and huge regional differences were reported (Nielsen et al., 2000). Calculated “true” prevalences were estimated between 0 and 91% depending on the region of sampling. In 1999, a similar study was carried out comprising 2709 herds in Southern Jutland, South Jutland and Western Jutland indicating that 75-90% of those herds were infected (unpublished data). These estimates were all based on the assumption that either a herd was infected or it was not. Any “latent” paratuberculosis infection would potentially be enough to give a positive status to a given herd. Estimates of individual-level prevalence depend on the definition of paratuberculosis. Because the current definitions of paratuberculosis are not consistent with the results obtained from the available diagnostic tests and the diagnostic tests are evaluated based on other definitions of “paratuberculosis animals”, estimates of individual-level prevalence are even harder to obtain than herd-level estimates. Estimates on the cow-prevalence in a dairy region using such traditional methods suggested that 5% of the cows were infected – based on an apparent prevalence of 11% (Nielsen and Agger, 2000). However, these prevalence estimates must be considered biased because potentially faulty assumptions have been used.

Aetiology

The aetiological agent of paratuberculosis is *M. avium* subsp. *paratuberculosis*, previously named *M. paratuberculosis*. Two other *M. avium* subspecies currently exist: *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* (Thorel et al., 1990). For differentiation of *M. avium* subsp. *paratuberculosis* from the two others, detection of

the specific insertion sequence IS900 by molecular techniques currently is the only option (McFadden et al., 1987a; 1987b; Green et al., 1989; Collins et al., 1990). Therefore, detection of this element by polymerase chain reaction is considered to be the definitive test for the identification of this organism. The other two *M. avium* subspecies also appear to have one or two unique sequences (IS901 and IS902) although these are not always present (Kunze et al., 1991; Moss et al., 1992). The use of the *M. avium* subsp. *paratuberculosis* “unique” IS900 identifier to assure specific tests may not be as unequivocal as first thought. IS900-like sequences have been demonstrated in two strains of *M. avium* that was not *M. avium* subsp. *paratuberculosis* strains isolated from clinically normal animals (Cousins et al., 1999). Thus, 100% specific identification seems difficult.

Immunology and pathogenesis

Infection with *M. avium* subsp. *paratuberculosis* is usually through oral uptake of faecal contaminated feed or raw milk in which the bacteria have been shed. In utero transmission is also a possibility (Seitz et al., 1989; Sweeney et al., 1992b). *M. avium* subsp. *paratuberculosis* is an obligate intracellular bacterium, hence cell-mediated immunity is the primary immunological response. How the bacteria enter intestinal tissues is still not fully elucidated but the following mechanism has been suggested: In the ileum, M-cells take up the bacteria, which are subsequently transferred to sub- and intraepithelial macrophages (Momotani et al., 1988). With the phagocytosis of the mycobacteria by macrophages, the immunological reaction has commenced. The macrophages are activated and a series of events can potentially occur, many of which are not fully described and understood. However, one of the two main immunological responses occurs: the cell-mediated T_H1-lymphocyte dominated reaction or the humoral T_H2-lymphocyte dominated reaction (Mosmann and Coffman, 1989; Waters, 2001). Briefly, the T_H1-lymphocyte response induces a cell-mediated immune-response with production of, among other cytokines, interleukin-12 (IL-12) and interferon- γ (IFN- γ). This cell-mediated response is characterised by activation of macrophages. While T_H1-lymphocytes are produced, differentiation of CD4⁺

lymphocytes into T_H2 cells is being suppressed. This suppression is mediated by the specific cytokines, e.g. IFN- γ . If the suppression is lifted, T_H2-lymphocytes will induce a humoral immune response. The humoral immune response is characterised by B-lymphocyte activation and production of immunoglobulins (antibody production). The differentiation into T_H2-lymphocytes is mediated by the cytokines IL-4, IL-6 and IL-10. These cytokines suppress T_H1-lymphocytes. Thus, in general the response will be dominated either by T_H1- or T_H2- lymphocytes. The T_H1-dependent macrophage activation is necessary for an effective elimination of the intracellular mycobacteria to take place since these are not available for the antibodies produced during the T_H2-dominated response. The mycobacteria also have a special capability of avoiding CD8⁺ T-cells (killer T-cells), because the mycobacteria are able to “hide” within the macrophages. Thus, degradation by cytotoxic CD8⁺ T-cells does not necessarily occur.

The two types of immune reactions are essentially in an equilibrium in which the factors determining dominance are not quite understood. The equilibrium will be shifted towards either one or the other type of immune response. Initially, the cell-mediated immune reaction dominates and the immune system is able to control the infection. Effective immunity to intracellular bacterial infection often requires the lysis of the infected cells as well as killing of the invading pathogen. Whether total elimination of the causative agent is possible in *M. avium* subsp. *paratuberculosis* infections is unknown and knowledge of the mechanisms of killing are mostly based on murine studies on *M. tuberculosis* (e.g. Schorey et al., 1997; Stenger et al. 1998). However, during the cell-mediated immune response, the number of *M. avium* subsp. *paratuberculosis* is, if not eliminated, kept at a low level in the infected animal. Evidence of a cell-mediated immune response can be measured through detection of lymphokines secreted from T_H1-cells, e.g. IFN- γ and IL-1. T_H1-cells also help B-lymphocytes in the production of IgM, IgA, IgG₂ and small amounts of IgG₁. During the cell-mediated immunological reactions the humoral response with production of antibodies is depressed, but the infection is still ongoing (Bendixen, 1977; Chiodini, 1996). Presence of CD8⁺ T-cells appears to block the signal from $\gamma\delta^+$ -T-cells to upgrade the CD4⁺-T_H2-population, which should otherwise lead to the production of

large amounts of antibodies (Chiodini and Davis 1992; 1993). Transition to the T_H2 -dominated humoral immune reaction may occur at a certain time. The basis for these transitions is still speculative, but one suggested mechanism is the dominance of $\gamma\delta^+$ -T-lymphocytes in calfhod as these T-cells together with $CD8^+$ T-cells are responsible for the proliferation of peripheral blood mononuclear cells in response to paratuberculosis antigens (Chiodini and Davis, 1992; 1993). This dominance in calfhod would explain the latency of infection in calves. In humans with *M. tuberculosis*-infections, genetic diversity of IFN- γ -receptors has been implicated in differences in the control of the immune responses (Altare et al., 1998).

Emergence of the immune response to paratuberculosis has been described most often in experimental infections. The immune response in natural infections, with all possible infection stages, apparently has not been described thoroughly. However, the timing of disease events in natural settings is pivotal in inference drawing on many aspects of a chronic disease. Therefore, the dynamics known from experimental studies may serve as a starting point. Here, studies indicate that cellular immunity may be detected 1-2 months after infection whereas the humoral immune function potentially can occur 10-17 months after infection (Lepper et al., 1989).

During the cell-mediated immune reaction, tuberculoid granulomas are formed in the infected tissues (mainly ileum). These granulomas are dominated by macrophages packed with mycobacteria. Later, with the T_H2 -lymphocyte dominance, lepromatous granulomas are seen. The granulomas are characterised by higher numbers of mycobacteria in the macrophages constituting the granulomas (Clarke, 1997). The severity of the pathological changes seems to be consistent with the high numbers of mycobacteria in the macrophages (Buergelt et al., 1978) but cows can also experience clinical disease following development of tuberculoid granulomas (Clarke, 1997). Data on the epidemiological distribution of pathological changes and their correlation with clinical findings and the immune responses are sparse and most often, severe cases of paratuberculosis dominate the literature. The events leading to the shift from the T_H1 - to the T_H2 -lymphocyte dominance are unknown. The dynamics of the shift in the equilibrium are also unknown as is the reversibility of the immune response though

it is generally thought that once T_H2-dominance has occurred, the reaction apparently does not reverse again (Waters et al., 2001) because effective immunity is induced by T_H1-dominance only (Chiodini, 1996). The timing of the shift is not known either. Whether a fixed period from infection to T_H1-dominance to T_H2-dominance is present is not known. Nevertheless, it is often assumed that T_H1-responses can be detected in calfhood shortly (i.e. a few months) after infection and T_H2-responses can be detected in adulthood with a fixed likelihood of a diagnosis no matter how old the animal is (if just being a cow²), but currently available literature does not suggest fixed timing of these events (Lepper et al., 1989; McDonald et al., 1999). Also, the theory of a fixed incubation time for paratuberculosis is in many instances self-contradictory. This is due to the chronic nature of the disease, possible latency of infection, and, though usually assumed to be in calf hood, the unknown time of infection. Thus, the dynamics of the infection in a natural setting covering all potential stages of infection and disease have yet to be described relative to the routinely applied diagnostic tools.

Diagnosis of paratuberculosis

Diagnosis of paratuberculosis follows the patterns of pathogenesis. Two main diagnostic patterns can be followed: techniques that detect the agent and techniques that detect the immune responses (reviewed in Nielsen et al., 2001). Isolation of *M. avium* subsp. *paratuberculosis* by traditional culturing from faeces can take 2 to 4 months (Whipple et al., 1991). Faster techniques have been developed (Cousins et al., 1995; Grant et al., 1998; Whittington et al., 1999) but these are still labour-intensive and require that excretion of the bacteria takes place at the time of sampling. Detection of an immune response could be performed by detection of antibodies e.g. using enzyme-linked immunosorbent assays (ELISA) or similar techniques. Alternatively, newer methods such as detection of IFN- γ are options. However, prior to detection of the immune response, the reactions have to have occurred. Because paratuberculosis is chronic and the time of infection usually is unknown, a major obstacle in obtaining a correct diagnosis is time. The analytical sensitivity of the test applied is another

² Cow is defined here as an animal that has had her first calf

obstacle. According to the pathogenesis, correlation of bacterial load and immune responses is time-dependent. Because the dynamic of the immune responses and the dynamic of the bacterial load and potential shedding do not necessarily follow the same pattern or time course, evaluation of the analytical sensitivity is difficult as the sample material often is defined based on some gold standard of infection such as detection of bacteria. Actually, considering the pathogenesis, the amount of analytes (the things being measured, e.g. bacteria or antibodies) differs with stage of infection. Some infections may never end with disease or losses to the farmer to a degree that it may be worth considering. Some diagnostic tests may therefore be adjusted in analytical sensitivity to ignore such reactions in order to maintain a reasonable specificity.

The analytical specificity is another issue that has to be considered. Cross-reactions to other mycobacterial infections are possible, either because the mycobacteria detected are not *M. avium* subsp. *paratuberculosis* or because the immune responses detected are caused by antigenic stimulation of non-*M. avium* subsp. *paratuberculosis*-like antigens. The degree of the false-positive reactions due to such cross-reactions is difficult to evaluate because neither the analytical sensitivity nor the analytical specificity can be fixed in a study. When the sensitivity is increased, the specificity will decrease.

Objectives of the present work

The diagnosis “paratuberculosis” depends on how paratuberculosis is defined. Paratuberculosis is defined based on the purposes of the diagnosis. Ultimately, important consequences to paratuberculosis infection in a herd are:

- negative influences on the individual animal ultimately decreasing the welfare of the animal (due to diarrhoea, emaciation etc.),
- production loss affecting the farmers economy,
- expenditures to treatment and prevention,
- transmission to other susceptible animals.

Thus, tests that can be used as proxies to establish the likely occurrence of these consequences are desired. Mathematics can be used to determine such probabilities. However, the purpose of the testing needs to be defined to provide a correct use of the mathematical figures emerging. Averaging the test performance for all purposes may result in in-efficient use of the tests. An example of hereof is provided by the recommendations of the National Johne's Working Group (NJWG)/ United States Animal Health Association (USAHA) (Anon., 2002a). The NJWG/USAHA base their test evaluation upon one definition of paratuberculosis. Subsequently, the test evaluations are extended to comprise all paratuberculosis definitions and all purposes. However, the test evaluation is only valid for a narrow disease definition.

To avoid possible misinterpretations, a statement of the purpose of a diagnosis is a requirement prior to concluding on a diagnosis. Also, separate test evaluations for separate definitions of paratuberculosis are required. However, because paratuberculosis is a chronic disease and no definitive tests exist to provide a final diagnosis for all purposes, an alternative would be desirable. The hypothesis of the present thesis is:

'Purposive interpretation of diagnostic information from diagnostic tests for various definitions of paratuberculosis can increase the value of the traditional tests, antibody ELISA and faecal culture.'

The purposes of the thesis are to give an epidemiological evaluation of the usefulness of the diagnostic information obtained from laboratories and use the information to study occurrence and dynamics of paratuberculosis in dairy cattle. New definitions of paratuberculosis will be suggested to provide for "cow-types" that can be used in decision support systems to facilitate decisions on actions for farmers and other decision makers, depending on their purpose with the diagnosis 'paratuberculosis'.

Chapter 2. Evaluation of diagnostic tests

Introduction

The aim of Chapter 2 is to introduce and evaluate the diagnostic tests used in the thesis. The chapter consists of three manuscripts. Paper I is included to provide an example where the “consequence” of paratuberculosis could pertain to transmission since the reference test detects bacteria in faecal samples. With a biased sampling method, an apparently superior accuracy of the test can be obtained. The cows studied cannot be considered representative of all cows infected with *M. avium* subsp. *paratuberculosis*. Paper II deals with the problem of defining study objects without this bias, namely through use of no-gold standard methods for evaluation of diseases that have stages of latency (Enøe et al., 2000). Here, ELISA and faecal culture are evaluated simultaneously without putting more weighting on one or the other. With the simultaneous evaluations it is also possible to show point estimates of the performance of the faecal culture when there is a shift in the level of antibodies. This can be extended further to drawing inferences from the faecal culture test on populations based on their antibody level.

In paper III the variability of the ELISA is given to demonstrate important components of variability and the need to control these in other studies. The paper serves more as a technical documentation for other papers rather than as an independent scientific contribution on inferences of laboratory tests from a veterinary point of view.

Integration of the knowledge obtained from the papers is done in Chapter 4 and will not be dealt with further within this chapter except for what is said in the specific papers.

Due to restrictions from the publishers of the journals in which paper 1, 2 and 5 have been published, these papers are not present in this PDF. The papers can be found in:

Paper 1:

Nielsen, S. S., Houe, H., Thamsborg, S. M., & Bitsch, V. (2001). Comparison of two enzyme-linked immunosorbent assays for serologic diagnosis of paratuberculosis (Johne's disease) in cattle using different subspecies strains of *Mycobacterium avium*. *Journal of Veterinary Diagnostic Investigation*, *13*, 164-166.

<http://jvdi.org/cgi/content/abstract/13/2/164>

Paper 2:

Nielsen, S. S., Grønæk, C., Agger, J. F., & 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.

DOI: 10.1016/S0167-5877(01)00280-X

Paper 5:

Nielsen, S. S., Grohn, Y. T., & Enevoldsen, C. (2002). Variation of the Milk Antibody Response to Paratuberculosis in Naturally Infected Dairy Cows. *Journal of Dairy Science*, *85*, 2795-2802.

<http://jds.fass.org/cgi/content/abstract/85/11/2795>

PAPER I

Comparison of two enzyme-linked immunosorbent assays for serological diagnosis of paratuberculosis (Johne's disease) in cattle using different subspecies strains of *Mycobacterium avium*

S.S. Nielsen, H. Houe, S.M. Thamsborg, V. Bitsch

Journal of Veterinary Diagnostic Investigation, 2001, **13**, 164-166.

Paper II

Maximum-likelihood estimation of sensitivity and specificity of ELISAs and faecal culture for diagnosis of paratuberculosis

S.S. Nielsen, C. Grønbaek, J.F. Agger, H. Houe

Preventive Veterinary Medicine, 2002, 53, 191-204

Paper III

**Variance Components of an Enzyme-linked Immunosorbent Assay
for Detection of IgG Antibodies in Milk Samples to *Mycobacterium
avium* subspecies *paratuberculosis* in Dairy Cattle**

S.S. Nielsen

Submitted to *Journal of Veterinary Medicine, series B*

**Variance Components of an Enzyme-linked Immunosorbent Assay for
Detection of IgG Antibodies in Milk Samples to *Mycobacterium avium*
subspecies *paratuberculosis* in Dairy Cattle**

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With 1 figure and 1 table

Summary

Milk samples from 120 cows were tested up to 10 times in an enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to *Mycobacterium avium* subsp. *paratuberculosis*. The purpose of the study was to estimate variance components of the assay attributable to laboratory factors using mixed model theory. Because of significant interaction between the between-run, between-day and between-plate variables, the ELISA-plate variable was nested in run-number and run-number was nested in day-number. The nested variable accounted for 72% of the laboratory variability ($p=0.0025$), whereas the intra-plate variability accounted for only 0.05% of the laboratory variability ($p=0.36$). Therefore, it was concluded, that the intra-plate variability could be ignored whereas the variability from the combined run-day-plate variable should be considered in any analyses based on the ELISA.

Introduction

Paratuberculosis is a chronic infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) (Chiodini et al., 1984). The infection has been described in a variety of animal species of both ruminant and non-ruminant origin, but the infection is most often associated with economic losses in ruminants. In Denmark, the infection is fairly prevalent in the Danish dairy cattle with approximately 50% of the herds infected (Nielsen et al., 2000) and 5-13% of the cows infected in some areas (Nielsen and Agger, 2000). Precise estimates of the prevalence of the infection are not available because of a variety of diagnostic problems. These include: a) cross-reactive antibodies to other closely related bacteria, especially *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*; b) lack of definitive proof of infection (lack of a gold standard) because *MAP* are obligate intracellular pathogens and can be present in an infected animal in minute amounts without being detected and perhaps even without yielding clinical disease; c) a cell-mediated immune response is expected to be the primary response, eventually followed by non-protective humoral immunity (Lepper et al. 1989; Stabel, 2000); d) chronicity of the infection with a typical incubation period of 1-3 years, perhaps longer (Whitlock and Buergelt, 1996); e) variable probability of having *MAP* antibodies with different cow characteristics (Nielsen et al., 2002b). Because of these factors potentially affecting the efforts to establish the diagnosis 'paratuberculosis' and because different farmers have different purposes with the final diagnosis, there is a need to come up with clear definitions that may help the farmer in the process making the diagnosis 'paratuberculosis' for the a specific purpose. Based on information from different studies (Nielsen et al., 2002a; Nielsen et al., 2002b; Nielsen et al, J. Dairy Sci.) different and dynamic antibody concentrations in different 'cow types' could be suggested (Fig. 1). The four cow types are: a) non-infected; b) infected with a cell-mediated immune response controlling the infection; small amounts of antibodies will be present because of an equilibrium between humoral immunity and cell-mediated immunity; c) and d) infected cows losing control of the infection at some time point. This could be either before or after they become cows (as defined by their first calving). To be able to detect small concentrations of antibodies,

the analytical sensitivity has to be high. However, we still have the complicating factor with cross-reacting antibodies, which have not been accounted for in Fig. 1, and which are not easily determined. Thus, if the detection limit should be maintained at a low level we have to make some compromises on the diagnostic specificity. Also, it is important to be able to differentiate between the unspecific reactions and laboratory variation. This has not been described with the enzyme-linked immunosorbent assay (ELISA) used in the above-mentioned studies. The variability used in these studies has been used to draw inferences on the ‘cow types’ in Figure 1. Therefore, the ELISA has been optimised to increase the analytical sensitivity. However, using the ELISA in this way also requires sound use of the laboratory variability and therefore knowledge on this has to be obtained.

The objective of this study was to determine the intra-laboratory variability due to within-run variation and between-run variation. The diagnostic performance have been described elsewhere (Nielsen et al., 2002a) and it was not the purpose to determine this.

Materials and Methods

Origin of samples

Samples from two herds were selected. From one herd, *MAP* had been detected on a regular basis for a number of years and this herd was classified as a “high-prevalence herd” (herd H). In the other herd, *MAP* had never been detected even though culture from faecal samples from all cows had been carried out 3 times during the past 2 years. This herd was classified as a “low-prevalence herd” (Herd L) since milk samples from some cows had previously shown to have significant ($p < 0.05$) probability of being infected, and some of the cows present in the herd were known to origin from a herd where *MAP* had been detected. In these two herds, all lactating animals were sampled through the monthly milk-recording scheme. From Herd L, samples from 62 cows were collected and from Herd H samples were collected from 58 cows. Using this sampling strategy it was assumed, that infected cows from different stages of infection along with different concentrations of antibodies would be

present. Of the cows from herd H, 44 cows had been faecal culture negative on one or more samplings, 4 cows had never been cultured, and 10 cows had been faecal culture positive on at least one sampling during the past 2 years. In Herd L, 38 cows had been faecal culture negative on 1-3 samplings and samples from 21 cows had never been cultured.

The milk samples collected were treated with bronopol (with a bactericidal effect) and methylblue at the sampling according to standard procedures. At arrival at the laboratory, the samples were centrifuged, the fat fraction removed, and the samples were frozen below -18°C for later testing.

Diagnostic test

An ELISA was developed based on a commercially available antigen (Allied Monitor, Fayette, Missouri, USA) and adapted for milk testing as previously reported (Nielsen et al., 2001; Nielsen et al., 2002a). This antigen is designated '*M. paratuberculosis* Strain 18' and it has previously been described to be a *M. avium* subsp. *avium* strain (Chiodini, 1993). Therefore, the analytical specificity of the antigen could be expected to be lower for this antigen than for a *M. avium* subsp. *paratuberculosis* strain. However, this was investigated in a different study (Nielsen et al., 2001) and no apparent difference was found, so it was decided to continue the use of this antigen.

Briefly, the diagnostic method was performed as follows: Polysorp microtiter plates (Nunc, Roskilde, Denmark) were coated with the antigen in a $2\ \mu\text{g/ml}$ solution (diluted in 0.1 M carbonate buffer, pH 9.6). The coated plates were left for 3 days at 5°C . Subsequent to unfreezing, the milk samples were diluted 1:2 in *M. phlei* (Allied Monitor) whereby the most unspecific antibodies were absorbed (Yokomizo et al., 1985). The absorbed samples were left overnight at 5°C . On the following day, the coated plates were washed five times in phosphate-buffered saline (pH 6.8) with 0.05% Tween₂₀ (PBST₂₀). The absorbed samples were then added to the coated plates ($100\ \mu\text{l}$ per well) and the plates were incubated to the following day at 5°C . On the subsequent day, splashing the content followed by 5 repeated washings with PBST₂₀

emptied the plates. To each well, 100 μl of a 1:2,000 dilution of peroxidase-labeled goat anti-bovine IgG (H+L) (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) was added. The plates were incubated 1 hour at 20°C and washed again 5 times with PBST₂₀. Ortho-phenylene diamine (OPD) (Kem-En-Tec, Copenhagen, Denmark) was diluted in citrate buffer (pH 5.0) to a concentration of 300 $\mu\text{g/ml}$ and 100 μl of solution was supplemented with 80 μl H₂O₂. One hundred microliter of this solution was added each well, and the chemical reaction was stopped with 100 μl 0.5 M H₂SO₄ per well when the colour development in the positive controls visually had peaked. Reading of the OD-values was done using an ELISA-reader with a 492-nm filter and a 620 nm-filter as reference.

Sample set-up

To test the between-day, between-run and between-plate variability, each sample was tested on three different days and at two different runs on two of those days. Also, to assess intra-plate variation, each sample was tested in duplicate in each plate. In total, each sample had the potential of being tested 10 times in 5 different ELISA plates. Unfortunately, for some of the samples there was only sample material for some of the ELISA-plates. Because of this reduction, 4 samples were tested only 6 times, 9 samples were tested 8 times and 107 samples were tested 10 times, a total of 120 samples tested 1166 times which were used for the statistical analysis.

Statistical analysis

A mixed model approach was used to analyse the data. In normal fixed effects models it is assumed that the variances for all observations are the same. This assumption is often violated due to various clustering effects whereas in the mixed model the covariance can be specified for random effects thus minimising the overparameterisation induced in a fixed effects model (Brown and Prescott, 1999). Therefore, a mixed model approach was used to analyse the data through analysis of variance using the Mixed procedure in SAS (Littell et al., 1996) based on the principles suggested by Singer (1998). The basic model used was:

$$Y_{ijklmn} = \beta_0 + \bar{C}_{.j} + \bar{T}_{.k} + \bar{P}_{.l} + \bar{R}_{.m} + \bar{D}_{.n} + U_{0klmn} + E_{ijklmn}$$

where

Y_{ijklmn} was the log transformed value of OD-values for a sample tested in test no. T in ELISA-plate P in run no. R on day D where the sample originated from cow C .

$C_{.j}$ = the fixed effect of the cow;

T_{ik} = the random effect of the test number in the ELISA plate;

P_{il} = the random effect of ELISA-plate;

R_{im} = the random effect of run number;

D_{in} = the random effect of day number;

U_{0klmn} = the group mean for the random effects;

E_{ijklmn} is the random error,

and where the random effects (RE) and E_{ijklmn} were assumed independent, identically distributed normal with mean 0 and variance components σ_{RE}^2 and σ^2 , respectively. The variance-covariance structure of all random effect matrices were the unstructured type.

Major interaction terms of the laboratory effects (all cross-products of the variables test number, plate-number, run number, day) were evaluated and if significant, the test number was nested in plate number, plate number in run number and run number was nested in day number. The model was evaluated using the likelihood ratio test (at $p=0.05$) and Akaike's Information Criterion (AIC) to obtain the model with the best fit. The above-mentioned assumptions on the distribution and independence were also evaluated.

The variance components of the random effects were evaluated using the restricted maximum likelihood and the results were used to estimate the sources of variation of the ELISA test. Normally, re-test of a sample is done when the difference between the duplicates is more than 0.100 OD-values. It was assessed whether this would change the results.

Results

The statistical analyses showed, that an interaction between plate-number, test day and run number existed whereas the other interaction terms were insignificant. Therefore, ELISA-plate was nested in run number, which was nested in test day. The resulting variance components are presented in Table 1.

Evaluation of the residuals showed that these were not normally distributed by Kolmogorov-Smirnovs test of normality. However, visual inspection of the results indicated, that these tests were insufficient and the result was influenced greatly by a number of outliers. Removing 14 outliers (based on the standardized residual criterion) and repeating the analysis did not change the estimates much (model 2, Table 1) whereas the residuals were now normally distributed according to the normality test. A plot of the predicted versus the residuals indicated that the observations were identically and independently distributed.

Usually, the sample from one cow is retested if the duplicate set-up of the sample differs by 0.100 OD-value. Sixteen duplicates had to be retested if this strategy was followed. Exclusion (model 3, Table 1) of retest samples did not change the estimates either.

Table 1. Variance components from a model to predict the ELISA response to *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. The model was based on 1166 samples from 120 animals from two herds sampled in September 2001.

Model and variance component (VC)	Covariance Estimate	Standard error	% explained by VC	z-test for significance (p-value)
Model 1 (All observations)				
ELISA-plate (Run(Day)) ¹	0.1517	0.05408	72%	0.0025
Test number	0.000102	0.000284	0.05%	0.36
Residual	0.05746	0.002583		< 0.0001
Sum of variation	0.20926			
Model 2 (Outliers excluded)				
ELISA-plate (Run(Day)) ¹	0.1448	0.05150	80%	0.0025
Test number	0.000019	0.000120	0.01%	0.44
Residual	0.03698	0.001675		< 0.0001
Sum of variation	0.1818			
Model 3 (Retest samples excluded)				
ELISA-plate (Run(Day)) ¹	0.1495	0.05330	73%	0.0025
Test number	0.000101	0.000280	0.05%	0.36
Residual	0.05456	0.002453		< 0.0001
Sum of variation	0.20416			

¹ELISA-plate(Run(Day)) = The nested effect of ELISA-plate in run number in test day.

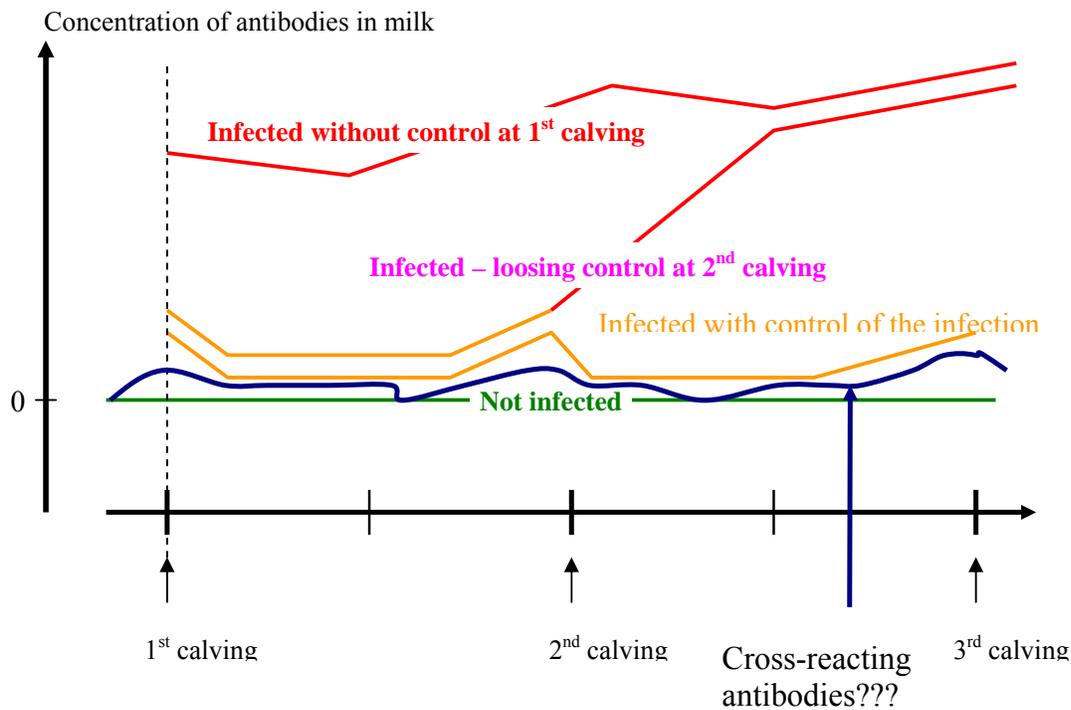


Fig. 1. Schematic representation of the theoretical dynamics of antibodies in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*. A probable effect of cross-reacting antibodies is also included.

Discussion

Establishment of the various contributors of variation and their relative importance is necessary to determine areas on where to put greater focus. It is also important to determine if the variation has a size, which renders action necessary. In this study it was determined, that the major variability of the present ELISA is located in the combined between-day-between-run-between-plate variable and that this variation could account for 72% of the total laboratory variability. This was 15% of the total variation in the data, where 80% of the variation could be attributed to the cow identity (data not shown). The intra-plate variability was, however negligible, with only 0.05% of the total variability. These findings affirm our general impression of the test that some between-plate variability does exist. The between-plate variability should be considered when interpreting the ELISA in general and if the ELISA should be used for routine diagnosis a smaller degree of variability would be preferable for ease of interpretation.

Significant between-day laboratory variability is not a one-stand occurrence in routine laboratory practice. In some tests such variability can be a huge problem, in others be insignificant. In research-oriented work the variability can be dealt with through appropriate statistical models. However, in the routine diagnostics, insignificant variability would be desirable. More routine among the laboratory staff on the specific test could be a requirement in some cases. If the problems would be solved in the present case is unknown.

Because of the variability described, it is not easy to determine a fixed cut-off, where any sample could be determined antibody positive or antibody negative at any concentration. Doing so with samples with know antibody concentration would provide an option, but the variability of these samples would be expected to be of the same magnitude as that of the samples described here, and the problems of cross-reactive antibodies still would not have been overcome. Preliminary analyses in other studies have indicated a difference in background colouring based on various cow characteristics (data not shown). To deal with such variability these factors would have to be elucidated further.

Mixed effects models are not generally used in test validations studies. However, one of the major advantages using mixed effects models is that it is not the level of antibodies that is modelled but it is the variation, which is modelled (Brown and Prescott, 1999). Modelling with mixed effect models therefore precludes artificial variance inflation due not the specific level of samples. Therefore, mixed effects models have a huge potential in test evaluations.

Based on the findings in this study it can be concluded that: the present ELISA presents with significant between-run variation, which should not be ignored. However, the intra-run variability has a magnitude that can be considered negligible.

Acknowledgements

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Chapter 3. Timing and dynamics of the immune response

Introduction

The aim of chapter 3 is to describe cow-time factors that are of importance to the ELISA reading and thereby interpretation. Paper IV describes the differences in odds of being ELISA positive in a serum ELISA and a milk ELISA for cows in different parities and at different stages of lactation. The data for the study in Paper IV are based on cross-sectional data. Paper V describes the dynamics of the ELISA values in different types of cows, where knowledge of faecal culture is also included. The gain by repeated samples is demonstrated and the difference in timing of the onset of immune responses is illustrated. Integration of the results with other information obtained in the present thesis is done in Chapter 4.

PAPER IV

**The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by
parity and stage of lactation**

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The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation

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Abstract

Two cross-sectional studies were carried out to determine the enzyme-linked immunosorbent assay (ELISA) response to *Mycobacterium avium* subsp. *paratuberculosis* by cow characteristics and stage of lactation. One of the studies (referred to as ‘milk-group’) used milk samples from all lactating cows (n=7994) in 108 Danish dairy herds. The other study (referred to as ‘serum-group’) used serum samples collected from all cows (n=5323) in a subset of 72 herds from the 108 herds. These samples were analysed using a similar ELISA for detection of antibodies.

The results from the ELISAs were interpreted with two cut-off values as the optimal cut-off value is not known, and as several levels are recommended to be used in practice. The results showed that the probability of being ELISA-positive was 2-3 times lower for cows in parity 1 relative to cows in other parities using both milk and serum ELISA. At the beginning of the lactation the probability of being positive was highest in the milk ELISA. In the serum ELISA the odds of being positive was highest at the end of lactation. The findings are important in the interpretation of ELISA results at cow-level with a subsequent tentative diagnosis and correction for parity and stage of lactation should be considered when providing a diagnosis of paratuberculosis. Some issues related to the pathogenesis are also discussed.

Keywords: ELISA; paratuberculosis; cattle-microbiological disease; antibody pattern

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the causative agent of the chronic disease paratuberculosis (also known as Johne's disease) of cattle and other ruminants (Chiodini et al., 1984). The incubation time is of varying duration, but in general cattle do not develop clinical signs of disease before 2 years of age (Whitlock and Buergelt, 1996). In some infected animals, clinical disease never develops, either because they are culled before the disease is manifested, or, as some suggest, because some infected cattle are able to clear themselves of the infection (Bendixen, 1978). One indicator of latent or subclinical infections could be changes in immune responses (Lepper et al., 1989). *M. avium* subsp. *paratuberculosis* is an intracellular pathogen that initially elicits a cell-mediated immune response. This is usually assumed to take place at the earliest, at 1-10 months of age. The cell-mediated immune response may then be followed by a non-protective humoral immune response at 10-17 months of age, though many infected animals do not show a clear humoral immune response before 33 months of age (Lepper et al., 1989). The humoral immune response is dominated by the cytokines produced by T_H2-cells. The key factor responsible for the shift to a humoral immune response remains to be identified, but at all times, a base-line level of antibodies (perhaps below the detection limit of most tests) is present.

Some commercial enzyme-linked immunosorbent assays (ELISAs), e.g. Parachek[®] (CSL Limited, Parkville, Victoria, Australia) and Herdchek[®] (IDEXX Laboratories, Westbrook, Maine, USA), use single cut-off values for inferring whether an animal is infected, and most studies undertaken to study risk factors for paratuberculosis have also used a single cut-off value to denote paratuberculosis positive animals (e.g. McNab et al., 1991; Jakobsen et al, 2000; Johnson-Ifeorlundu et al., 2000). These cut-off values are established to gain as high a specificity as possible, often referenced as more than 99% specific (Collins et al., 1991) in order to minimise cross-reactions by antibodies stimulated by the ubiquitous *M. avium* subsp. *avium*. The chronic nature of the disease, the varying incubation time, differences in

farm-level presence of *M. avium* subsp. *avium* and differences in immune responses of animals, however, suggest that the results from the ELISAs should be interpreted with more than two outcomes. Also, animal characteristics and perhaps farm-specific interpretation should be considered and optical density (OD) values should be corrected for these factors. The varying incubation time and the fact that some infected cows never become clinically diseased (Clarke, 1997) indicate the necessity of using several levels of interpretation of the ELISA results given different objectives; for example, to identify infected animals or to identify animals that are more likely to shed mycobacteria or develop clinical disease.

Production and concentration of antibodies in milk and serum, especially IgG₁ and IgG₂, have been described in many studies. However, these studies focus mainly on the production of IgG necessary for transferral of passive immunity to the offspring (e.g. Shearer et al., 1992; Guy et al., 1994) rather than studying pathogen specific changes with time. The latter have to our knowledge not been determined with paratuberculosis but have been described with mastitis pathogens (Caffin et al., 1983; Caffin and Poutrel, 1988). Most studies indicate a concentration effect on IgG at the beginning of lactation in milk with a concomitant decrease in serum IgG (Dixon et al., 1961; Williams and Millar, 1979; Guidry and Miller, 1986). Few studies have investigated the variation across the entire lactation (Klobasa et al., 1977; Guidry and Miller, 1986) and in these studies, total IgG was studied rather than pathogen specific IgG.

The objective of our study was to determine whether ELISA response to *Map* varies by cow characteristics and stage of lactation.

2. Materials and Methods

2.1. Herds and animals

Throughout the text, reference to infection and paratuberculosis is according to the following definitions: Infection was any condition where entrance of

Mycobacterium avium subsp. *paratuberculosis* (*Map*) or related bacteria might have caused a rise in antibodies detected by the diagnostic test used. It was assumed infection persists for life. Further subdivision encompasses *Map* infection and “other mycobacterial infections yielding a *Map*-like immune-response” detectable by ELISA and indistinguishable in ELISA from *Map*. Paratuberculosis (including both clinical and subclinical) was any condition where infection, as defined above, gives rise to any unfavourable condition, e.g. diarrhoea, weight loss or loss of milk yield.

Data for the cross-sectional study originated from two data sets, referred to here as ‘milk-group’ and ‘serum-group’ collected in the period August 1999 to February 2000. The herds in the ‘serum-group’ were a subset of the herds of the milk-group so details on the ‘serum-group’ are given when appropriate.

The animals in the ‘milk-group’ were from a region with approximately 260 dairy herds in Southern Jutland, Denmark, where the Danish Dairy Board initiated a study in 1998 (Andersen et al., 2000). Of these herds, 108 gave their permission for studies on infectious diseases (*Escherichia coli* O157, salmonellosis, mastitis related to *Streptococcus uberis* and paratuberculosis). Milk samples (n=7994) were collected once from all lactating cows in these herds, from the routine milk production scheme. On arrival at the Cattle Health Laboratory³, the samples were centrifuged, the fat fraction was removed and the skim milk frozen for later testing. The animals in the ‘serum-group’ were from a subset of 72 herds from the 108 herds. Twenty-eight of the herds were selected based on the highest prevalence of high-level ELISA response in the milk samples. The remaining 44 herds were identified based on a first-sign-up-for-the-project basis. Samples were collected in un-stabilised blood tubes, and upon arrival at the laboratory, the samples were centrifuged, the blood clots were removed, and the serum fractions were frozen for later testing.

The size and composition of the herds were as follows: minimum: 21 cows; 25th percentile: 55 cows; median: 65 cows in milk-group, 70 cows in serum-group; 75th percentile: 89 cows in milk-group, 86 cows in serum-group; maximum: 229 cows. The breed composition of the animals was as follows: a) Red Danish: 208 cows in the

³ The Cattle Health Laboratory, Danish Dairy Board, Ladelundvej 85A, DK-6650 Brørup, Denmark

‘milk-group’, 121 cows in the ‘serum-group’; b) Danish Holstein: 6488 cows in the ‘milk-group’, 4325 cows in the ‘serum-group’; c) Danish Jersey: 858 cows in the ‘milk-group’, 664 cows in the ‘serum-group’; d) Danish Red-and-White: 18 cows in the ‘milk-group’, 17 cows in the ‘serum-group’; e) Crossbreed: 422 cows in the ‘milk-group’, 196 cows in the ‘serum-group’.

2.2. ELISA methods

The serum samples in the ‘serum-group’ were tested using a serum ELISA previously described (Nielsen et al., 2001). The milk samples in the ‘milk-group’ were tested using the same ELISA, but slightly modified, i.e. the antigen was used in a concentration of 2.0 µg/ml instead of 1.0 µg/ml, conjugate was used at the dilution of 1:2,000 instead of 1:4,000 and dilution of the samples was 1:2 in *Mycobacterium phlei* in the milk ELISA only. Serum was diluted 1:10. Each sample was tested in duplicate. The results from the tests were given as raw optical density (OD) values. The raw OD-values were adjusted with a standard sample used on all ELISA-plates (4 wells containing the standard sample per plate) by subtracting the average OD-value of the standard sample from the average OD value of the sample under consideration. These corrected OD values (OD_C) were used for the statistical analyses.

Table 1

Distribution of cows by ELISA-category and cut-off points from 108 herds (‘milk-group’) and 72 herds (‘serum-group’).

Category	Cut-off		Number of samples in category	
	Milk ELISA	Serum ELISA	Milk-group	Serum-group
0	$-4 < x \leq -0.030$	$-4 < x \leq 0.345$	6272	4028
1	$-0.030 < x \leq 0.600$	$0.345 < x \leq 0.884$	1605	1145
2	$0.600 < x \leq 4$	$0.884 < x \leq 4$	117	150

2.3. Statistical methods

The ELISA reaction was evaluated using logistic regression analyses with the GLIMMIX procedure in SAS[®] version 8. Initially, the OD_C-values were divided into three categories: 0, 1 and 2. Cut-off point 1 was the OD_C where the validity of the diagnostic tests was optimised, i.e. where the point of the receiver-operating characteristic (ROC) curves was closest to the point (0,1) in a ROC-diagram when evaluating the test. Cut-off-point 2 was the point where the ROC-curve was steepest, i.e. the specificity of the test was optimised (Nielsen et al., 2002). The estimated sensitivities (Se) and specificities (Sp) at these two cut-off points for each ELISA were: Se_{Milk,1}=0.817 and Sp_{Milk,1}=0.745; Se_{Milk,2}=0.391 and Sp_{Milk,2}=0.964; Se_{Serum,1}=0.652 and Sp_{Serum,1}=0.721; Se_{Serum,2}=0.161 and Sp_{Serum,2}=0.961 The specific cut-off values and the number of samples for each of the two tests are given in Table 1.

Subsequent to the production of three ELISA levels, descriptive statistics were carried out. Because the number of animals of some breeds was small and because there was no apparent difference into the number of cows at each ELISA-level for breeds other than Jersey, these were all combined into one group. Jerseys, however, have been described to have a higher risk of paratuberculosis (McNab et al., 1991) and higher ELISA reaction (Jakobsen et al., 2000). Therefore, breed was categorised into Jersey and Non-Jersey. All cows were categorised into one of three parity groups: Parity 1, 2 and >2, and categorised into one of four stages in lactation: 1-2 weeks, 3-12 weeks, 13-28 weeks and 29-44 weeks. Data on all cows more than 308 days in lactation were omitted because of apparently different ELISA-patterns and because we wanted to make sure they were not classified in the wrong lactation. Pritchett et al. (1991) reported that the IgG₁ concentration was influenced by volume of milk, so the milk yield on the day of sampling was included in the analyses to control for this effect. The milk yield variable was standardized based on the predicted milk production using the model:

$$M_{ik} = b_0 + b_1 * \frac{D_k - 60}{245} + b_2 * \frac{D_k - 60}{245} + P_i \quad (1)$$

where M_{ik} is the standardised milk production on the k^{th} day (D_k) in lactation for cows in parity P_i ; b_0 is the estimated mean milk production at day 60 in milk, b_1 is the coefficient for the change in milk production from day 1-59 in milk, b_2 is the coefficient for the change in milk from day 60-308 in milk, and P_i = the effect of the i^{th} parity (parity was defined as 1=1, 2=2, 3=3 or greater).

The two datasets were initially analysed with proportional hazard polytomous regression using the logistic procedure in SAS[®]. However, the assumption of proportional hazards was not met (Hosmer and Lemeshow, 1989, pp. 217-233). Therefore, the data were analysed comparing each of the ELISA-levels 1 and 2 to level 0, separately, using the GLIMMIX macro in SAS[®], with herd as a random effect. Given herd was non-significant, logistic regression using the Logistic procedure in SAS[®] was used instead. The explanatory variables evaluated were: Jersey/ Non-Jersey, stage of lactation, parity and 1st order interactions between these three variables. If interaction was demonstrated between parity and stage of lactation, the latter was nested in parity. The regression models were created using backward elimination, using the likelihood ratio test at the 95% level of confidence as the cut-off for inclusion in the model. The resulting parameter estimates were used for calculation of population odds ratios and probabilities for stage of lactation, and parity when controlling for milk yield as a fixed effect (milk-group only).

3. Results

The descriptive statistics yielded a distribution of samples in each of the 3 categories of the response-variable as given in Table 2. All results of Jerseys are omitted because the interaction between parity and Jersey was significant and there were too few Jerseys to fit a reasonably precise separate model for that breed.

For each of the datasets, a final logistic regression model was fitted at each of the ELISA cut-offs (1 and 2), including stage of lactation and parity. Herd was not found

significant (at $p=0.05$) using the likelihood ratio test, thus fixed effect models were used. The first model was:

$$\ln\left(\frac{Y_{ijk}}{1-Y_{ijk}}\right) = U + P_i + S_j + b_1 * MD_k + e_{ijk}, \quad (2)$$

where Y_{ijk} = ELISA-result (positive=1, negative=0 at low cut-off);

U = the underlying background level of the ELISA;

P_i = the effect of the i^{th} parity (parity was defined as 1=1, 2=2, 3=3 or greater);

S_j = the effect of the j^{th} stage of lactation (stage of lactation was defined as 1 = weeks 1-2, 2 = weeks 3-12, 3 = weeks 13-28, 4 = weeks 29-44 post partum);

MD_k = milk production (kg) on the k^{th} day in lactation minus the expected milk volume on the k^{th} day of sampling according to equation 1;

b_1 = regression coefficient for MD_k ;

e_{ijk} = a random residual component;

MD_k was included only in the analyses of the 'milk-group'.

No significant interaction between parity and stage of lactation was revealed for the 'milk-group' at the low cut-off (Table 3). Parity 2 and greater cows were approximately 3-4 times more likely to be ELISA-positive than parity 1 cows. The odds of being positive in the first two weeks of lactation were almost twice those during weeks 29-44.

The second model was fitted for the 'milk-group' at the high cut-off (Table 4). In parity 2 and higher, the odds of being ELISA-positive were approximately twice those of being ELISA-positive in parity 1.

The logistic regression of the 'serum-group' at the low cut-off yielded an interaction between parity and stage of lactation. Therefore, stage of lactation was nested in parity (Table 5). In general, there was an increase in the odds of being ELISA-positive with increasing parity. However, during the first weeks of lactation, the odds were generally lower than the odds at the end of the previous parity (Fig. 1).

The model fitted for the ‘serum-group’ at the high cut-off for the ELISA is presented in Table 6. Parity 2 and greater cows were significantly more likely to be ELISA-positive relative to parity 1 cows with odds for being ELISA-positive of 2-2.5 times higher than in 2nd and higher parity cows.

Table 2

Frequency distribution^a of cows by parity, stage of lactation and ELISA category after data editing (no Jersey cows and no cows >308 days in lactation). Cross-sectional sampling of milk samples from 6090 cows in 101^b herds and serum samples from 3796 cows in 66^b Danish dairy herds.

	Milk ELISA category ^c			Serum ELISA category ^c		
	0	1	2	0	1	2
Parity 1						
Weeks 1-2	76 (78%)	21 (22%)	0 (0%)	57 (92%)	4 (6%)	1 (2%)
Weeks 3-12	609 (93%)	48 (7%)	1 (0%)	403 (91%)	36 (8%)	3 (1%)
Weeks 13-28	705 (94%)	46 (6%)	3 (0%)	467 (87%)	59 (11%)	10 (2%)
Weeks 29-44	680 (89%)	76 (10%)	9 (1%)	381 (82%)	72 (16%)	10 (2%)
Parity 2						
Weeks1-2	34 (63%)	17 (31%)	3 (6%)	38 (83%)	7 (15%)	1 (2%)
Weeks 3-12	324 (78%)	88 (21%)	4 (1%)	195 (73%)	63 (24%)	8 (3%)
Weeks 13-28	511 (78%)	128 (20%)	12 (2%)	245 (69%)	102 (29%)	8 (2%)
Weeks 29-44	398 (75%)	125 (24%)	5 (1%)	186 (62%)	98 (33%)	14 (5%)
Parity >2						
Weeks 1-2	46 (59%)	32 (41%)	0 (0%)	37 (77%)	10 (21%)	1 (2%)
Weeks 3-12	414 (73%)	146 (26%)	10 (2%)	223 (67%)	99 (30%)	10 (3%)
Weeks 13-28	678 (77%)	190 (22%)	12 (1%)	375 (66%)	165 (29%)	26 (5%)
Weeks 29-44	446 (70%)	188 (29%)	5 (1%)	262 (69%)	106 (28%)	14 (4%)

^a) In a few cases, the frequencies do not add up to 100% because of rounding.

^b) ELISA category 1: Cut-off-point 1 < OD_{corr} ≤ Cut-off-point 2

^c) Some herds had only Jersey cows, hence the whole herd was omitted

Table 3

Logistic regression for ELISA antibody response at low cut-off for milk samples from 6090 cows in 101^a herds. The model included parity and stage of lactation^b.

Stratum	Regression coefficient	Standard error	OR (95% C.I.) ^c
Intercept	-2.197	0.086	-
MD [□]	-0.035	0.006	-
Parity			
1	0.000	-	1.00
2	1.148	0.095	3.15*** (2.62-3.79)
>2	1.354	0.089	3.87*** (3.25-4.61)
Stage of lactation			
Weeks 1-2	0.565	0.159	1.76*** (1.29-2.40)
Weeks 3-12	-0.196	0.087	0.82** (0.69-0.98)
Weeks 13-28	-0.356	0.081	0.70*** (0.60-0.82)
Weeks 29-44	0.000	-	1.00

^a Some herds had only Jersey cows, hence the whole herd was omitted

^b Difference in milk volume relative to expected milk volume (MD) was included as a fixed effect.

^c 95% C.I. = 95% Confidence interval.

P<0.01, *P<0.001

Table 4

Logistic regression for ELISA antibody response at high cut-off for milk samples from 6090 cows in 101^a herds. The model included parity^b.

Stratum S	Regression coefficient	Standard error	OR (95% C.I.) ^c
Parity			
Intercept	-5.349	0.284	
MD [□]	-0.135	0.020	
Parity 1	0.000	0.000	1.00
Parity 2	0.856	0.348	2.35* (1.19-4.65)
Parity >2	0.611	0.345	1.84 (0.94-3.62)

^a Some herds had only Jersey cows, hence the whole herd was omitted

^b Difference in milk volume relative to expected milk volume (MD) was included as a fixed effect.

^c 95% C.I. = 95% Confidence interval.

*P<0.05

Table 5. Logistic regression for ELISA antibody response at low cut-off for serum samples from 3796 cows in 66 herds^a. The model included stage of lactation nested in parity.

Parity and stage of lactation	Regression coefficient	Standard error	OR (95% C.I.) ^b
Intercept	-2.434	0.466	
Parity 1			
Weeks 1-2	0.000	0.000	1.00
Weeks 3-12	0.098	0.496	1.10 (0.42-2.91)
Weeks 13-28	0.521	0.484	1.68 (0.65-4.35)
Weeks 29-44	0.895	0.482	2.45 (0.95-6.29)
Parity 2			
Weeks 1-2	0.876	0.607	2.40 (0.73-7.89)
Weeks 3-12	1.423	0.487	4.15** (1.60-10.77)
Weeks 13-28	1.633	0.480	5.12*** (2.00-13.12)
Weeks 29-44	1.926	0.482	6.86*** (2.67-17.64)
Parity >2			
Weeks 1-2	1.221	0.579	3.39* (1.09-10.55)
Weeks 3-12	1.718	0.481	5.57*** (2.17-14.30)
Weeks 13-28	1.759	0.475	5.81*** (2.29-14.73)
Weeks 29-44	1.653	0.479	5.22*** (2.04-13.36)

^a Some herds had only Jersey cows, hence the whole herd was omitted

^b 95% C.I. = 95% Confidence interval

*P<0.05, **P<0.01, ***P<0.001

Table 6. Logistic regression for ELISA antibody response at high cut-off for serum samples from 3796 cows in 66 herds^a. The model included parity.

Stratum	Regression coefficient	Standard error	OR (95% C.I.) ^b
Intercept	-4.122	0.206	
Parity			
1	0.000	0.000	1.00
2	0.746	0.273	2.11** (1.23-3.60)
2>	0.898	0.251	2.46*** (1.50-4.01)

^a Some herds had only Jersey cows, hence the whole herd was omitted

^b 95% C.I. = 95% Confidence interval

P<0.01, *P<0.001

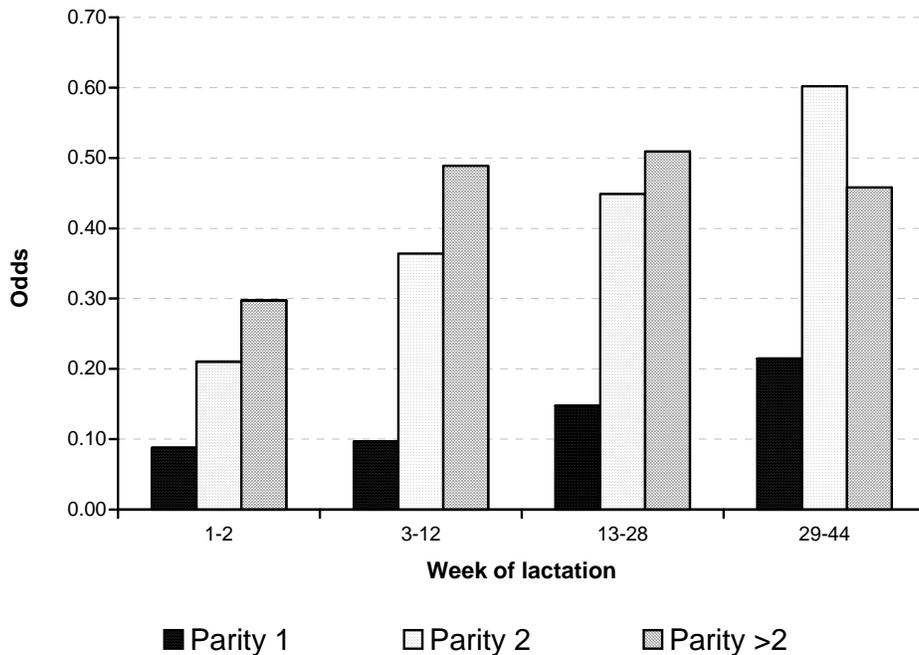


Fig. 1. Odds for being ELISA positive for cows from the 'serum-group' at the low cut-off point for cows in different parities at different stages of lactation in 3796 cows from 66 Danish dairy herds.

4. Discussion

Paratuberculosis is a chronic infection with a prolonged immune response. The primary immune response is a cell-mediated immunity, where production of antibodies occurs but is more or less suppressed. In some animals, a secondary humoral immune response will assist to increase the concentration of antibodies. The results in this study indicate, that although some cows are ELISA-positive (in either test and at either cut-off point) in the 1st parity, the probability of being positive is at maximum in parity 2 or higher. Also, the probability of being ELISA-positive is different across a lactation, with inverse patterns of probabilities between milk and serum indicating a change in antibody concentration throughout the lactation. This is only the case at low cut-off points, possibly indicating that cows with low antibody concentrations are infected but with a cell-mediated type of immune response. This is speculative and would have to be supported by a longitudinal study with repeated measures on the

same cows. Multiple response levels or ELISA-readings on a continuous scale would help solve this pathogenetic hypothesis.

From a diagnostic point of view it is important to recognise the differences in ELISA positives in different parities at different stages of lactation. The use of different cut-off points in the interpretation of ELISA results could resolve the issue if the ELISA is to be used in routine diagnostics.

In this study, the low prevalence of high-level responders calls for careful interpretation of the results. Similarly, the specificity of the tests are not 100% and cross-reactions with other mycobacterial infections are possible. Cows in this study did not have a definitive diagnosis of paratuberculosis with an agent detecting method. However, knowing the poor correlation between agent detection and immune response detection (Nielsen et al., 2002), such information would probably not change the conclusions but rather misclassify some cows due to the low sensitivity of agent detection methods. The low sensitivity of ELISA tests, in this study, would also misclassify some cows as negative even though they are infected. Therefore, the odds ratio estimates provided could as well be too low as too high.

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Due to restrictions from the publishers of the journals in which paper 1, 2 and 5 have been published, these papers are not present in this PDF. The papers can be found in:

Paper 1:

Nielsen, S. S., Houe, H., Thamsborg, S. M., & Bitsch, V. (2001). Comparison of two enzyme-linked immunosorbent assays for serologic diagnosis of paratuberculosis (Johne's disease) in cattle using different subspecies strains of *Mycobacterium avium*. *Journal of Veterinary Diagnostic Investigation*, *13*, 164-166.

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Paper 2:

Nielsen, S. S., Grønæk, C., Agger, J. F., & 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.

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<http://jds.fass.org/cgi/content/abstract/85/11/2795>

PAPER V

**Variation of the milk antibody response to paratuberculosis in
naturally infected dairy cows**

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Chapter 4. Discussion: Definition of paratuberculosis – Cow-typing and purposes of testing

Introduction

‘Paratuberculosis’ can be considered the disease⁴ entity, which may follow infection with *Mycobacterium avium* subsp. *paratuberculosis*. This condition is not easily determined in many situations. The main reasons are the nature and the dynamics of the infection and the variable validity of the diagnostic tests. The undesirable effects of the infection can be decreased animal welfare, production losses and perhaps decreased food safety, all of which are of different interest to different decision-makers, i.e. farm managers, agricultural politicians, food safety managers and others.

Techniques, time and tests are the main issues dealt with in the past 2 chapters of this thesis. Each component may contribute to the perception of paratuberculosis. The perception of paratuberculosis should be in agreement with the purposes of the decision-makers. Thus, techniques, time and tests are also needed when addressing the definition of paratuberculosis. The following is a discussion on implementing the knowledge from Chapters 2 and 3 for different control purposes with additional discussions concerning the implications of variability of the tests used to determine paratuberculosis status. First, a brief introduction of the use of diagnostic tests in control programs for infectious cattle diseases is provided. Then, a discussion linking the papers presented in the past chapters is given along with some critical remarks of some potential drawbacks of the papers are listed. This discussion addresses handling of technical and biological variability. Finally, a proposal for defining paratuberculosis cow-types based on ELISA-results are given in order to extend the use of ELISA for different purposes.

⁴ General disease definition according to Dictionary of Veterinary Epidemiology (Toma et al., 1999): Noncompensated perturbation of one or several functions of an organism.

Use of diagnostic tests in control programs

Diagnostic tests are crucial in many infectious disease control and surveillance programs. However, the tests still need to be interpreted in combination with other information to obtain purpose-related information applicable in the control program (Bitsch and Rønsholt, 1995). In Denmark, examples of control and surveillance programs include surveillance of enzootic bovine leukosis (Anon., 2000) and infectious bovine rhinotracheitis (IBR) (Anon., 1999) based on bulk tank-milk and slaughterhouse blood samples; and eradication and surveillance of bovine virus diarrhoea (BVD) virus based on bulk tank-milk and other sampling strategies (Anon., 2001). In the BVD control scheme, the tests constitute a major factor for monitoring the success of the control program. They are also important in detecting new infections in herds previously free of the infection and in the status of individuals being traded. The BVD-tests for individuals are not stand-alone tests. The major diagnostic advantages of BVD and IBR infections are the acute nature of the immune responses and easily interpretable tests. Information on herd or animal factors may need to be added to the information obtained from the laboratory tests to make conclusions according to one's desires but the laboratory tests are cornerstones in the Danish BVD control scheme. In the scheme, different animal "types" are present: "Acute infections" temporarily harbouring both BVD virus and BVD antibodies but ultimately only BVD antibodies; "Immunotolerant persistently infected calves" with BVD virus and no BVD antibodies, etc. The test results and combinations of different laboratory test results are handled differently for different purposes: some for monitoring the development (hopefully success) in the program; some for detection of new infections; and some for detection of infection-transmitting animals, i.e. different approaches for different purposes (Anon, 2001).

Paratuberculosis infection is different in some aspects. The infection is chronic and in some infected cows, clinical disease may never develop (Chiodini, 1996; Whitlock and Buergelt, 1996). This is also due to the short production life of Danish dairy cows. The tests used for in this thesis are far from being 100% sensitive and specific as has been shown in the past chapters and elsewhere. This variability related

to the lack of valid test responses is from two major sources: biological and technical. The biological variability reflects the complex pathogenesis and will be discussed later.

Technical variability

The technical variability is a reflection of the variation of the laboratory tests irrespective of the origin of a random sample. Two different test methods have been used in the papers in this thesis, a bacteria culturing method and an immune test, an indirect antibody ELISA. The culture method is optimised to obtain a high analytical sensitivity without a loss of viable bacteria through the process of decontamination and laboratory processing. The result is fairly simple to interpret: presence or absence of the bacteria. Prior optimisation of the bacteriological culture with information concerning herd or animal characteristics is not immediately possible. Quantification of the bacterial load could have been performed in the present studies and thereby perhaps have added more information on the severity of shedding. Separation of the material presented in more categories (e.g. +, 2+, 3+ and 4+ as provided by the laboratory based on the number of colony forming units) would have produced more categories to provide this information. However, since repeated sampling from the same cows in another study gave varying results without apparent systematic effects (data not shown), subdividing the results of the bacterial culture on the background of colony-forming units was judged meaningless at the time. Hence, samples were classified as culture positive and culture negative only.

For the ELISA, post-laboratory optimisation of the analytical sensitivity is possible. Or to be more exact, a combination of mathematical procedures and technical optimisation renders further interpretation of the ELISA test possible because information is kept through keeping variation in the low end of the reading scale of the ELISA rather than deleting the information. In any test system, the components of variability should be known to some extent in order to take necessary steps in eliminating or controlling significant variation. Having introduced a test system where we have attempted to increase the analytical sensitivity allowing higher laboratory

variation, we need to know the important reasons for variation in the test system in order to control them in other statistical analyses. To address the components of variability, the study described in Paper III was carried out. Based on that study, it was concluded that there was an effect of the ELISA-plate in which a given sample was tested in on a given day. Such variation is expected when using the ELISA technique and has been reported in other studies, also on paratuberculosis (Sweeney et al., 1995). Therefore, we have to in some way address the plate-to-plate variation. However, even though this variation has been recognized, no single standard method of dealing with the variation in statistical analyses of ELISA data is generally applied. Standard methods have been suggested in a publication of *Officine International des Épizooties* (Wright et al., 1993), but the mathematical basis for some of these must be considered vague, and in some instances even erroneous, because biological and technical variation is mixed. Two of the listed methods for reporting ELISA readings, raw optical density (OD) values and corrected OD values (OD_C), are used in the work in the present thesis. A third, normalised OD-values, could also have been considered. The latter method is recommended by Jacobson (1998) for consensus purposes, but a ‘statistically more correct standard method’ has not been determined. The raw OD-value is the reading from the ELISA-reader. The corrected OD-value is calculated by subtracting a value of a negative control from the raw OD-value. The negative control should be representative of the negative population in which the test is used. This can be difficult to obtain, especially if no good reference test exist. If such representativity cannot be obtained the impact of the including the negative control should be assessed or the raw OD-value should be used if possible – with subsequent appropriate statistical corrections for those explanatory factors found significant. The normalised OD-values are corrected for a factor determined on the basis of the expected versus the observed OD-values for a defined positive reference standard. Using the normalised OD-values, information may be lost in the correcting-phase as they assume that the reference standards variation is fixed. Random variation is attributed to the test sample only. In Papers I, II and IV, OD_C was used as the basis for the response variable in the statistical analyses. OD_C can be used as an approximation of plate-to-plate level and

thus general systematic laboratory variation. However, it assumes that the negative control operates with only systematic effects and does not allow for random error of laboratory variability. In an optimised statistical setting, use of raw OD-values would therefore seem more appropriate. However, the statistical tools are not yet developed to handle all situations. Therefore, both the raw and corrected OD-values have been used in the present studies. The effect of using one or the other has not seemed to be important in a few pilot studies made (data not shown). In the papers presented in this thesis it was therefore decided to use the raw OD-values when deemed possible and then include correction for laboratory variation in the statistical models. As shown in Paper III, high between-run variability was seen. One explanation already given was lack of continuing experience by the laboratory technicians. Other explanations are influences of assay reagents, time and temperature as are usual causes of variation in ELISA-procedures (Jacobson, 1998). However, these effects are usually not seen when recalculating to normalised or corrected OD-values, but because we used raw OD-values, the effect might seem more severe. Reporting the variation is rare, but Sweeney et al. (1995) also experienced significant variation, and dealing with the variation is thus necessary.

Biological variability

If the technical variability can be controlled or dealt with, we can focus on, from a veterinary point of view, the more interesting parts, namely the biological variation. The biological variation relates primarily to the pathogenesis. In a simplified scenario, the course of paratuberculosis could be: Infection – cell-mediated immunity/pathological lesions possible – humoral immunity/pathological lesions frequent/diarrhoea possible (for more details and other plausible scenarios, see Chapter 1). With the long incubation period, the need for inclusion of aspects of the pathogenesis in the evaluation of test results is even more important than in many other test-disease relations. When evaluating antibody ELISAs, the immune-response part of the pathogenesis is the most important part. The ELISAs used in the present study measure both IgG₁ and IgG₂ (see Paper I). Thus, using a static view (at a specific

point in time) of the immune-response, each ELISA (serum and milk) has two important cut-off points: 1) differentiation between no immune-response and cell-mediated immunity (primarily IgG₂); and 2) differentiation between cell-mediated immunity and humoral immunity (primarily IgG₁). In the scenario where animals do not move between disease stages, the most widely used approach is to establish a case definition of paratuberculosis to address the performance of tests used for testing. A thorough post-mortem examination including histopathology or isolation of *Mycobacterium avium* subsp. *paratuberculosis* from tissues is usually considered the best evidence of infection. However, these methods may not detect all infections. It is difficult to determine 'infection status', if none of the methods used are 100% sensitive. Whittington et al. (1999) recently provided a good example of this based on infected sheep. Neither radiometric cultures from faeces and tissues nor histopathology were able to detect all infected animals. Results presented by Whitlock et al. (1996) indicate, that a similar situation can be expected with bovine paratuberculosis, though some differences might be expected due to differences in sheep and cattle strains of *M. avium* subsp. *paratuberculosis*.

Exploring the ELISA based on known standards

In Paper I, 'paratuberculosis' is defined as cows shedding of bacteria (faecal culture positive) for estimation of sensitivity of the ELISA. Cows from an area assumed to be free of paratuberculosis was used for estimation of the specificity of the ELISA. However, this case definition is rather narrow as we only achieve knowledge on the sensitivity of the ELISA when analysing samples from animals shedding bacteria. Shedding of bacteria does not necessarily provide knowledge on infection stage as shedding can be intermittent or even just be a case of bacteria passing through the gastro-intestinal tract of uninfected animals (Sweeney et al., 1992a). Determining faecal shedding of bacteria may be the aim of one decision-maker but many other goals of testing may be desired by a decision-maker rather than just establishing whether an animal is 'shedding' or not. Also, knowing that paratuberculosis is a chronic infection, we also have to address dynamic aspects of the disease. Other

drawbacks of the study presented in Paper I are that the study have fairly few study objects to conclude that the ELISA is the optimal test for paratuberculosis testing. However, the ROC-curves are both significantly greater than 0.5 indicating that faecal culture positivity and ELISA-positivity are correlated. The microbiological procedures performed may be to insensitive to detect all shedders, thus only heavy shedding is detected which can result in a serious selection bias towards the case definition ‘shedding’.

Exploring the ELISA without known standards

Further exploration of the interpretation of the ELISA is required. First, a static population view is explored in Paper II. Basic issues of dynamic situations on cow and population level will be discussed later. In a situation, where a reference population cannot be obtained from the target population, and in a situation, where many disease stages are present, a latent class model seems appropriate in the exploratory phase rather than starting out with a case definition in order not to limit the exploration referencing known standards. Case definitions that are useful in practise may then be obtained subsequent to the analyses. In Paper II, a set of samples was collected to address evaluation of ELISAs and faecal culture from a latent class point of view. The intention of the sampling strategy was that the study objects were chosen to meet two requirements only: 1) they should be cows, i.e. have had their first calf; and 2) they should be from the target population. However, for sensitivity estimation, we need a sufficient number of true-positive animals sampled also. Therefore, additional strategies were also used to assure that the proportion of true-positive animals was increased without affecting the representativity of all stages of disease. Subsequent to the sampling, the resulting laboratory test results were analysed using a latent class approach, maximum likelihood estimation of sensitivity and specificity with the Hui-and-Walter method (Hui and Walter, 1980). The method requires for two tests, that a minimum of two subpopulations with different prevalences exist. This requirement is due to the fact that 6 parameters need to be estimated. To achieve enough degrees of freedom for the estimation, two populations (or put differently, two 2x2-tables) are

needed. The Hui-and-Walter method is really developed for dichotomous tests. Because we are really interested in the test performance in the target population, which is dynamic, and because multiple stages of disease exist in this population, we have to evaluate the ELISAs at multiple cut-off values to dichotomise the ELISA. We dichotomised the faecal culture for reasons mentioned earlier. However, the ELISAs may still contain much more information in the ordinal or continuous form. In the situation where there is no unique separation of antibody positive and antibody negative study objects, we could use a combination of latent class approach and receiver operating characteristic (ROC) analysis. This would lead to an exploratory analysis of the capabilities of both the ELISAs and the faecal culture. In the study in Paper II, a number of cut-off values was chosen as a starting point (Table 5 in Paper II). All cows were included in each of these calculations. This approach optimises the use of the size of our sample at each cut-off value. However, when we use latent class models, we need more study objects than in situations with known case definitions (Toft et al., 2002). However, the resulting estimates on the same sample are theoretically better. The sample size influences the width of any credible confidence intervals calculated and also the power of the study. When the difference in the prevalences between the populations is small, the effect on the width is greater than with a larger difference. A difference in prevalence that is based on a systematic factor is required to divide the target population into subpopulations for the estimations. Even a random difference would suffice but it could potentially be too small resulting in wide confidence intervals. We chose three dividers: Herd-size, postal zip-code and veterinary practice code number. Our only reasons to believe that a systematic differences in prevalence were that: herd-size have previously been described as a factor that may affect the prevalence of infectious cattle diseases (Braun et al., 1990; Gottschau et al., 1990; Nylin et al., 2000); differences in the advisory services given to farmers could give rise to a difference in prevalence between herds from one or the other veterinary practice; differences could just be due to geographical or other random factors. The apparent small differences in the appearance of the ROC-curves

may be due to different differences in prevalence among the subpopulations and are thus an expression of the uncertainty of the latent class model used.

In Paper II, the uncertainty associated with the parameter estimates are not given because it was not feasible at the time with the available computer programs and because the analyses should merely be seen as exploratory analyses of the performance of the tests. Calculation of the confidence intervals could have strengthened the study by illustrating the magnitude of the uncertainty associated with the maximum-likelihood estimates. However, what was more intriguing to figure out was the shape of the resulting ROC curves. Why do they appear very steep initially and later have a rather long course with occasional small increases? The ROC curves in Paper I appear different. First, they are very steep. Then they assume a fairly flat course. A plausible explanation is that more of the cows included in Paper I have end-stage paratuberculosis. In Paper II, the diversity of study objects is greater. Hence, there seem to be some disease stage parameters that need to be addressed. With a chronic infection, time is a parameter that requires thorough consideration. In Paper IV, we study the effect of time based on cross-sectional data. The results indicate that cow-time is an important factor in that both parity and stage of lactation are found significant factors that are worth considering as explanatory factors in the ROC analyses, either directly or by stratifying for those covariates. However, the computer programs to do so would require further development before doing it directly and simple stratification would make the number of study objects in each group too small for credible estimates. Thus, for the time being, the average estimates would have to suffice in the scenario of Paper II. It could be argued, that a major disadvantage of that study was that a definitive diagnosis for each cow could not be obtained. However, this seemed impossible considering the issues noted above and use of latent class models in such a situation would constitute a major motivation rather than a disadvantage.

Cow-time as a factor in ELISA results

In Paper IV, the primary conclusions were that the ELISA results were affected significantly by parity and stage of lactation. Because we only had one sample per cow in this study as in all previous, it should be considered to describe only static situations. More information about disease dynamics could be obtained from a study including several samples collected over time from each cow such as done in Paper V. This could also provide an insight into the dynamics of the antibody pattern of both faecal culture positive and faecal culture negative cows. The resulting analyses in Paper V showed that huge variation in cow profiles exists. Both among the culture positive and the culture negative cows, many kinds of predicted antibody profiles are seen. Analyses of technical variation were included in the statistical models and may therefore be ruled out as causes of the variable antibody profiles. This finding strongly suggests that latent class models should be used for evaluations of diagnostic tests for paratuberculosis. It also questions the validity of the ELISA as a potential tool for use in control of paratuberculosis, especially if the results of the ELISA are judged on a dichotomous scale without inclusion of knowledge on time factors. In many instances, ELISA positive cows may turn out faecal culture negative even though the ELISA results are persistently fairly high, and this undermines the use of ELISA to confirm positive faecal culture results. One way of determining the usefulness of ELISA results in actions against paratuberculosis is by estimating production losses associated with high, medium and low ELISA-values. In a study by Kudahl et al. (unpublished data) estimated milk production losses 0-305 days-in-milk were 496 kg energy corrected milk (ECM) for primiparous cows, 1318 kg ECM in second parity and 625 kg ECM in higher parities for one unit of standardised OD-values (standardised by time-covariates as described in this thesis). Smaller production losses were seen with standardised OD-values between 0 and 1 OD-units.

Population versus cow-level interpretation

Returning to Table 5 in Paper II. What does it mean to display the data at multiple cut-off values and draw inferences at some of these? In Figure 4.1a, the theoretical distribution of OD-values for the 3 immuno-populations, i) non-infected; ii) infected with cell-mediated immunity; and iii) infected with humoral immunity are displayed for a target population including both infected and non-infected animals. The figure shows a nice separation of the immuno-populations with only little overlap. However, in a dynamic setting, some animals may move from one immuno-population to another when going from one disease stage to another. The separation between the populations is probably presented too simple because the time component in moving from one disease stage to another is not included. Instead, the situation may be like in Figure 4.1b with more blurry borders, thus making multiple cut-off points necessary in exploring potential use of antibody ELISAs for different purposes and scenarios and for cows with variable probabilities of testing positive for faecal shedding. As mentioned earlier, huge uncertainty must be expected for the point estimates. However, using the serological profile of a population as an indicator of which immuno-population a specific cow belongs to and subsequently drawing inferences on

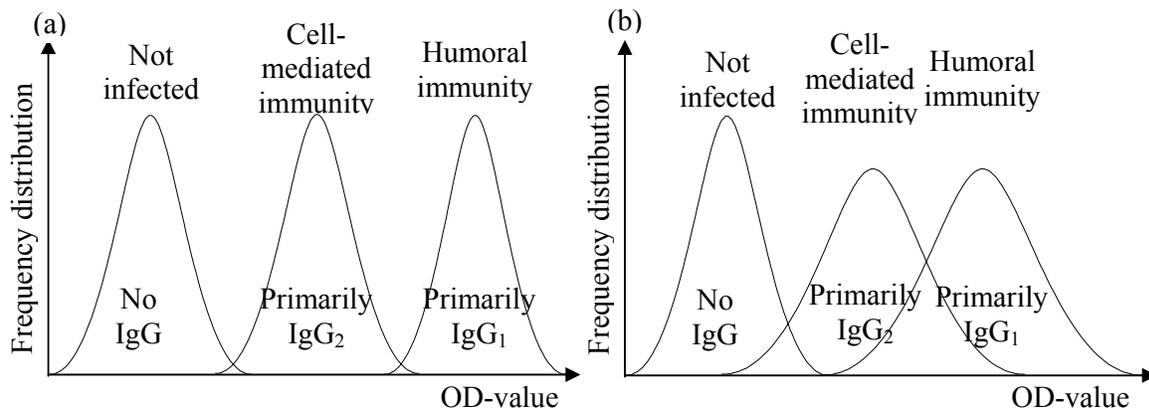


Figure 4.1. Theoretical distribution of OD-values for cows that are divided into distinct groups based on their immune response. (a) shows the static situation where it is assumed that cows going from one immuno-group to another are not present. (b) shows the dynamic situation which includes cows that are shifting immune-groups. In the non-infected cow, no immune-response is expected but the response in the ELISA depends on other factors. In the cow with the cell-mediated immune-response, IgG₂ is mainly responsible for the size of the OD-value. In the cow with a humoral immune-response, the OD-value is primarily due to IgG₁.

the performance of the faecal culture test does not yield surprising results compared to findings of others. For example, Whitlock et al. (1994) estimated a sensitivity of 29% for initial faecal culture for cows tested repeatedly in a four-year period. This could be compared to the sensitivities at cut-offs -0.087 (Se~20-25%) and -0.030 (Se~38%) from the present study, for cows expected to have a cell-mediated immune response. Higher sensitivity of faecal culture should be expected as disease progresses. Again, it should be emphasized that latent-class models are associated with greater uncertainty than models on cows with known disease status. It is also important to emphasize, that the ROC-curves based on the maximum-likelihood estimates are average population estimates. This means that the results from Paper II should be interpreted from a population point of view rather than an animal point of view. Therefore, the examples used above are to be interpreted for 'average cows', which do not exist in the real world. Using the average estimate assumes that the average cow is representative of a given immuno-population. And, it should be emphasised, that cows in our study were collected only once.

Which of the cut-offs should be selected and how would the test be optimised in a practical setting? First, the biological variation described should be included in the optimising process. Subsequently, a number of other criteria are necessary to address when optimising the diagnostic value of a test. Among those are the test outcomes false-positive (FP) and false-negative (FN), and the prevalence in the target population (the latter usually being the herd of origin). In order to assess the diagnostic value of a test, considerations to the FP and FN proportions are essential, especially if a cut-off value needs to be established to dichotomise a test result (Greiner et al., 2000). A thorough ROC-analysis could deal with this through optimising either the sensitivity, the specificity or both simultaneously, and then calculate the costs of FP and FN test results in each situation. The prevalence strongly affects the predictive value of the test result, as a positive test result is more likely to be a true-positive in a high-prevalence population, and a negative test result is more likely to be negative in a low prevalence population. Thus, knowledge on the prevalence and the FP and FN proportion is necessary to obtain. However, an even more important criterion for assessing the value

of a test is the aim of the testing. Because the Danish dairy industry and the individual farmers do not have a clear strategy on the testing for paratuberculosis, deciding whether emphasis should be on the FP proportion or the FN proportion is difficult to determine. Ultimately, a decision support system based on influence diagrams or related techniques would be required to determine consequences in a wider setting. A Bayesian approach with influence diagrams (Jensen, 1996) could be an option, but other simulation models could also address these issues. A false-positive could result in culling of valuable cows that are not easily replaced. However, in herds with many replacement heifers, this is not often a big problem. A false-negative could result in production loss and decreased animal welfare for the individual, risk of transmission of *M. avium* subsp. *paratuberculosis* to the herd-mates. The value of the latter is even more difficult to determine than just production losses. The weighting of false-positives relative to false-negatives should ideally change with prevalence.

Based on our findings, it can be concluded that the ELISA contains more information interpreted on a continuous scale, alone or in combination with faecal culture. Neither test can effectively rule out the use of the other. But both the biological and the technical variability need to be handled cautiously in order to use the test results strategically and with an interpretation according to the purpose of the strategy. In the following sections, definitions of cow types and purposes will be suggested. The cow types and listing of purposes are proposals for cornerstones in the initiation of a future voluntary control and eradication program for paratuberculosis in Denmark.

Definition of cow types

Progression of paratuberculosis infection in cattle (and thus the pathogenesis) is pivotal for inference making on the available diagnostic tests. Knowledge on the performance of the diagnostic tests in relation to infection stage and disease development therefore must be considered important. However, the long incubation period and the uncertainty about whether an infection will lead to a “consequence” (as listed in Chapter 2) in all infected cows, require that an epidemiological description of

paratuberculosis be made prior to inference drawing. ‘Epidemiological’ in this context refers to coverage of all cows in the infected population rather than just those that will be visually evident at a certain time-point. Both ‘infected’ and ‘clinically diseased’ animals can give reactions in the diagnostic tests. But different situations may require differences in the test interpretation i.e. whether the test is used for diagnostic, prognostic or surveillance purposes. Therefore, defining cows based on the expected disease development and test outcomes can be beneficial.

Thus, a description of the dynamic cow population would include three or four cow types (under the assumption that cure is not possible):

- a): Not infected.
- b): Infected, ‘controlling’ the infection (‘In-active infection’)
- c): Infected, initially ‘controlling’ the disease but losing control at a sudden point in time (‘Active infection’).
- d): Infected, not ‘controlling’ the infection (‘Active infection’).

By ‘control of the infection’ is meant the successful arrest of progression of disease by achieved effective immunity of a dominating cell-mediated immune response. Types b) and d) can may appear as static infections whereas type a) and c) are dynamic. Under the assumption that cattle are infected in calthood, only type c) needs to be considered dynamic. This assumption will be used in the following but the interpretation can be extended beyond this assumption. Thus, the static types can be generalised to three typologies: 1: Not infected; 2: In-active infection; 3: Active infection (Table 4.1). Both cows with Active as well as In-active infection may shed bacteria through different mechanisms. However, shedding is assumed much more prevalent in active infections, as described below. In Table 4.1, ‘controlling’ infection does not imply that shedding does not take place, simply that intermittent shedding is possible (Whitlock et al., 1994).

Interpreting the tests from a static point of view basically means that cross-sectional data can be used for the test evaluations. This has been done in Paper II where two ELISA tests and a test using faecal culture have been evaluated in a population that must be considered to include all possible stages of paratuberculosis

Table 4.1. Static paratuberculosis (ParaTB) cow types, with possible inclusion of dynamic elements of shedding.

Non-Infected	Infected			
	‘Controlling’ infection		Not ‘controlling’ infection	
	Non-shedding	Shedding	Non-shedding	Shedding
1	2		3	
ParaTB free	In-active ParaTB		Active ParaTB	

infection. Subsequent to the evaluations populations in which the tests can be used are defined. High antibody reactions, e.g. at a corrected optical density (OD_C) of 0.600 give a sensitivity of the faecal culture test of 0.65. Had the cut-off been lower, e.g. at an $OD_C=0.030$, the sensitivity would be only 0.38. The specificity remains 0.98 in both cases. However, from Paper IV we also know that the OD-values are strongly affected by parity and stage of lactation. Therefore, inclusion of these factors into the interpretation may strengthen the tests further, but how much has not been shown yet. This is left to future studies.

Transferring the knowledge from Paper IV to Table 4.1 now makes sense. Non-infected cows may be test-positive in some instances whether using faecal culture or ELISA as the testing tool. Cows with In-active ParaTB controlling the infection have a low level of antibodies. The concentration of antibodies depends on how the equilibrium has been shifted from cell-mediated immunity towards humoral immunity and the extent of IgG_1 and IgG_2 present. Shifts will usually only be expected to occur in cows that move from one disease stage to another. These shifts have currently been excluded from our explorations. However, some concentration mechanisms related to the milk-serum concentration of antibodies seem to exist. Generally, in the infected animal, there is some level of antibodies (minute amounts of one or both of IgG_1 and IgG_2). It is now assumed that this level on average is at $OD_C=0.030$ in Paper IV. Thus, for In-active ParaTB the sensitivity is 0.38. Cows with Active ParaTB generally are assumed to have higher levels of antibodies. This could be at $OD_C=0.600$ as suggested in Paper IV. The sensitivity of the faecal culture test would then be 0.65 on average. That the sensitivity is based on average values can be seen from Paper V where some cows with persistently high ELISA levels are persistently faecal culture

negative. The dependence of the ELISA on shedding of bacteria is not unequivocal. Paper IV shows that there is a relationship between the faecal culture test and the ELISA test. However, they are almost independent conditioned on disease, which can be seen from the small changes in the parameter estimates in Paper II. In Paper V it is seen that some cows that have been persistently antibody negative are faecal culture positive and vice versa. Probable explanations of this may be found in two of the assumptions made previously: 1) that cows are infected in calfhood and not as older animals; 2) that we are dealing with a static population. The first assumption is a usual generalisation of the transmission pattern (Chiodini et al., 1984; Sweeney, 1996). However, it has previously been shown that adults can be infected and develop pathological changes (Larsen et al., 1975) though some age resistance seems to occur (Hagan, 1938). No epidemiological reports on the extent and significance of adulthood infections relative to calfhood infections seem to exist. Still, in a heavily contaminated environment even older animals may be infected and subsequently shed bacteria which could lead to a positive test result in faecal culture with no concomitant antibody production due to a short incubation period. Shedding can also be due to passive transfer of *Mycobacterium avium* subsp. *paratuberculosis* through the intestinal system or false-positive results in the faecal culture test. The latter would usually be growth of non-*Mycobacterium avium* subsp. *paratuberculosis*-mycobacteria on the growth medium. Overall, the usually assumed obvious linkage between ELISA and faecal culture tests is not so obvious. This linkage results in the use of faecal culture to confirm ELISA results (Anon., 2002a; Anon., 2002b). The studies presented here links sensitivity of ELISA and faecal culture in cows in assumed early stages of disease, where both tests must be considered fairly insensitive. Thus, ELISA results may need to be interpreted on their own.

Faecal culture negative cows that are antibody positive could be the result of false-negatives in the faecal culture test or false-positives in the ELISA test. Both are known to occur (e.g. in Paper V). One solution is by determining losses in production and include 'production losses' as a complementary test in a decision support system.

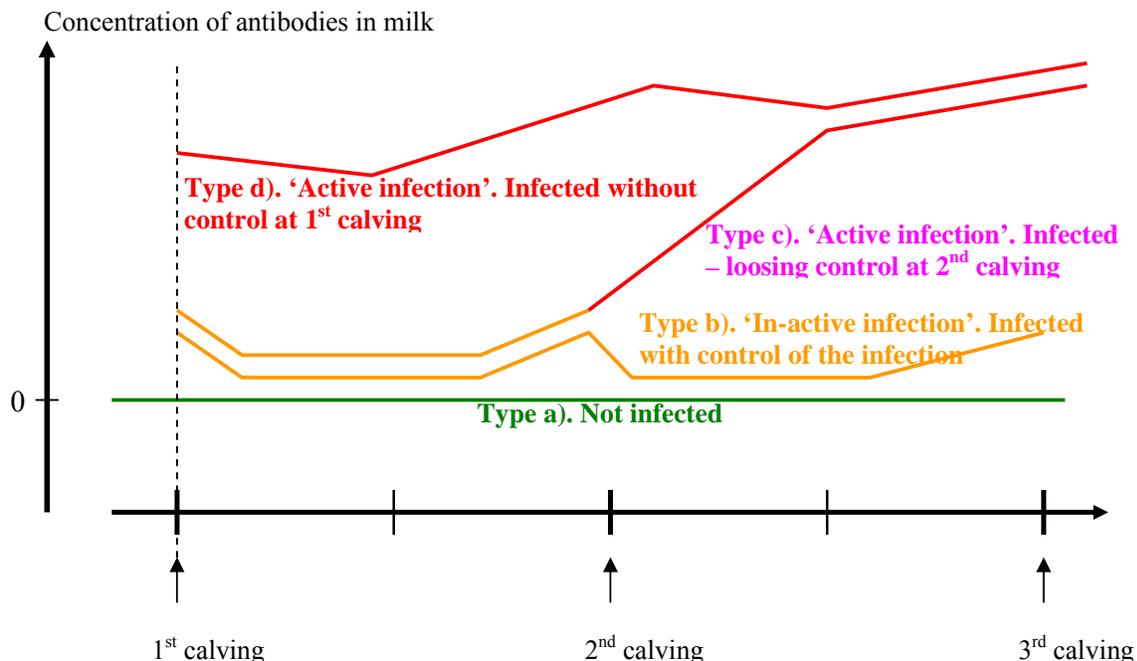


Fig. 4.2. Schematic representation of the theoretical dynamics of antibodies in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*. The appearance of the lines presented are based on the piece-wise linear regression models from Paper V, but smoothening of the lines would be required in a future development of the shape of the lines. For further explanation, see text.

If the combination ELISA-test + production loss is important, the faecal culture status should be irrelevant. This matter goes beyond the scope of this thesis.

Pursuing the dynamic aspect of paratuberculosis, Paper IV and V demonstrate not only a great variability in the ELISA response but also a very dynamic expression of the immune response. The status relative to shedding bacteria is of significant influence on the ELISA result. However, both in faecal culture negative and faecal culture positive cows, the variation is significant. Based on these findings, the findings in Paper II and the discussion above, the static cow types suggested above may be extended to dynamic cow types as illustrated in Figure 4.2. The lines represent each of the four different cow types a), b), c) and d) listed above and are hypothetical. However, they are intended to form the template for future interpretation on milk ELISA results as one branch in a testing scheme for paratuberculosis, the other branch being faecal culture testing. Cows that are shedding bacteria are not necessarily having ‘Active ParaTB’. But they may transmit the infective agent to other susceptible

animals. Combining the information in Fig. 4.2 and Table 4.1, provides a lot of information to draw inferences on specific cows given the purpose of the testing is known.

Purposes of testing

Different people have different purposes of using the laboratory tests. There are three main purposes from a veterinary point of view:

- i) diagnosis (i.e. confirmation that *Mycobacterium avium* subsp. *paratuberculosis* is causing in clinical disease (production loss, diarrhoea, weight loss or other undesired conditions)),
- ii) prognosis (i.e. prediction of a test result on the probability that *Mycobacterium avium* subsp. *paratuberculosis* is implicated in “clinical disease”),
- iii) screening (i.e. systematic surveillance to ascertain evidence of presence of *Mycobacterium avium* subsp. *paratuberculosis* that may at some time cause clinical disease).

The tests can thus be used for diagnostic or predictive (Choi, 1997) inference making but the sensitivity and specificity will be different depending on the purpose because the tests are not perfect. The sensitivity and specificity depend on stages of infection, which can partly (but not fully) be described by some animal factors.

The purpose of the testing and the aim of the tester must be defined prior to applying the test. Main categories of aims may include:

- reduction in prevalence of animals with clinical disease,
- reduction in prevalence of infected animals, and
- certification of freedom.

Table. 4.2. Proposed use of laboratory tests¹ for achievement of superior sensitivity without compromising specificity to unacceptable levels in cows for detection of paratuberculosis in cows. The proposal also includes priority of the tests with 1 being the test used as the primary tool, eventually supported by the test with priority 2.

Disease stage	Prevalence		
	Low – ‘Zero’	Mid	High
Early	1. ELISA	1. ELISA	1. ELISA
		2. FC	2. FC
Mid	1. ELISA	1. ELISA	1. ELISA
		2. FC	1. FC
Late	1. ELISA	1. FC	1. FC
	2. FC	2. ELISA	2. ELISA

¹ ELISA = enzyme-linked immunosorbent assay for detection of antibodies in milk or serum; FC = culture for detection of *M. avium* subsp. *paratuberculosis* in faecal samples

Combination of test purpose and aim of the tester should therefore be included in the planning of any control program for paratuberculosis. The two tests used in the present thesis, ELISA (both milk and serum) and culture of *M. avium* subsp. *paratuberculosis* from faecal samples, can be used for all test purposes but with variable predictive and diagnostic value. Table 4.2 provides a proposal for use of the tests used in this study for different situations in a voluntary control program on paratuberculosis. The table links prevalence and testing purpose. However, this linkage is actually through a combination of “Prevalence” and “Disease Stage”. In Table 4.3, examples of further elaboration of prevalence, disease stage and purposes are related to decision on test choice and priority. Other tests are not considered but the testing scheme can potentially be expanded to include these tests. Also, the testing scheme covers only adult animals. Because the two tests measure two very different and to some degree independent characteristics of the infection with *M. avium* subsp. *paratuberculosis*, they can be used either in serial testing or parallel testing with an almost full gain in specificity or sensitivity, respectively, without the loss of gain that can be expected from two tests that measure the same. However, the same feature (the

relative independence) makes them quite poor in confirming the diagnosis of the other as is recommended in some control programmes, e.g. in the USA (Anon., 2002a). This strategy mimics a desire to not find the disease, rather than leaving this decision to the decision maker ordering the test result. For prevalence reduction purposes, the strategy will be recommended in the initial phase, but a generalisation of this confirmation is not recommended. Unwanted consequences of such a generalisation can be seen for instance in the Dutch Johne's disease certification program, where herds assumed free of infection are not truly free (Weber et al., 2002).

Table 4.3. Examples of likely combinations of test choices and priority in a purpose-related testing scheme.

Purpose of strategy	Status in target herd and animals		Test choice priority
	Prevalence	Disease stage	
Reduce negative influence on individual animals	High	Early, mid, late	1. Faecal culture 2. ELISA
Reduce production loss affecting farmers economy	Mid	Mid, late	1. ELISA 2. Faecal culture
Reduce transmission	Mid	Late	1. Faecal culture 2. High ELISA

The strategies in Table 4.3 may be considered confusing, because they require thorough considerations in every step of categorisation. The cows are classified according to some unknown (maybe estimated) factors of the surrounding environment. An alternative to this complicated model is the one in Table 4.4. A simpler presentation is provided for inclusion in material for end-users. But ultimately, the interpretation of the diagnostic tests needs to be stringent to type the cows.

Table 4.4. Relations between alternative cow typing and purpose of strategy.

Purpose of strategy	Important cow types
Reduce negative influence on individual animals	Active ParaTB
Reduce production loss affecting farmers economy	Active ParaTB
Certificate related (economy)	Not infected
Reduce transmission	1. Active ParaTB 2. (In-active ParaTB)

Paratuberculosis is widespread in Denmark and fairly prevalent (Nielsen et al., 2000). The chronicity of the disease and the relatively low sensitivity of the tests for predictive purposes render detection of presence of *M. avium* subsp. *paratuberculosis* challenging. At a certain point in time, it is no longer challenging, because the prevalence is so high that late stage paratuberculosis is visible frequently as diarrhoeic cows scattered among other cows with diarrhoea. A clear linkage exists between the laboratory tests and the severity of disease (Whitlock et al., 2000), which makes purpose testing the only solution for establishment of a sound paratuberculosis program. Strategies to control infection in infected herds can be established with the existing tests, but the tests are not the only tools in eradication of the disease. Effective control of *M. avium* subsp. *paratuberculosis* should still include basic changes in management factors to reduce transmission of bacteria between animals. The tests may serve as success monitoring factors, but not as disease and infection eradicators on their own.

Chapter 5. Conclusions and perspectives

Testing alone cannot establish a control program, which will lead to successful control of paratuberculosis. Risk assessment at animal, herd, and regional level with subsequent changes in management procedures are required (Kennedy and Benedictus, 2001). Laboratory tests may serve as part of the toolbox for changes in management procedures. However, for tests to be included in a control program, they also have to be cost-effective in relation to the purpose of the testing.

In this thesis, an alternative to traditional test evaluation has been provided (Paper II). It is demonstrated how sensitivity and specificity of one test changes when the performances characteristics of the other test changes. Demonstrating bacterial shedding is easier in cows with high-level antibodies. It is concluded that the sensitivity of the faecal culture test depends on antibody level as a proxy of disease stage.

It is demonstrated, that the antibody level of a cow is affected by cow characteristics such as parity and stage of lactation (Paper IV). It is demonstrated that both faecal culture positive and faecal culture negative cows can obtain high ELISA-values (Paper V). It is demonstrated that low-level ELISA-values change with cow characteristics as do high-level values, but they apparently change differently for different cow-types (Paper V). The information obtained in the papers and the knowledge of the immunology and pathogenesis has been combined to create theoretic cow types in Chapter 4. The cow types are: I) Not infected; II) In-Active ParaTB; and III) Active ParaTB with extensions into a dynamic types as well. These cow types are suggested for use in a future Danish control program if such a program is established.

The cost-effectiveness of the diagnostic tests has not been evaluated. However, on-going work evaluating the effect on production in the cow-types suggested will eventually support further development of test strategies. Simulation studies including the cow-types for different purposes of testing is required.

The ELISA technique is not currently being used routinely for paratuberculosis testing in Denmark. In other parts of the world, e.g. Australia, USA and The

Netherlands, it is widely applied. However, in some instances, the reliability is questionable for single-animal test results and confirmatory tests are carried out on ELISA-positive animals (Anon., 2002a; b; Bulaga and Collins, 1999). Apparently, there is no reason for this in general, if the presence of the infection in a herd has once been established by a definitive test. Actually, confirmatory tests have such poor sensitivity in certain situations that such a strategy is not recommendable.

The suggested cow-types can be incorporated in a voluntary control program in Denmark in that they should form the basis for strategies on culling animals and monitoring of individual herds. Purpose-based strategies need to be elaborated simultaneously in close co-operation with the herd veterinarian. With no legislation, the different aims (e.g. animal welfare, economic losses, increased food safety) of having one or more infected cows are so far the only motivators that should drive the farmers into controlling the infection. The strategies should be tried out in selected herds and succeed prior to establishment of regular control programmes. Also, further studies on the pathogenesis of paratuberculosis, for instance time-to-event analyses to determine relationships between bacterial shedding and cow-types or factors determining shift from inactive to active infection need to be conducted.

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