UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

RISK ASSESSMENT CONCEPTUAL MODEL OF OCCURRENCE OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN UNPROCESSED BEEF AND BOVINE MILK

DIOGO FILIPE PEREIRA MARQUES

CONSTITUIÇÃO DO JURI

Professor Doutor Fernando Jorge Silvano Boinas

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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TRAINING DESCRIPTION

During a 8-month curricular training period the candidate was enrolled at the Faculty of Veterinary Medicine (FMV-UTL) – Technical University of Lisbon at the Department of Epidemiology & Economics and Veterinary Public Health (01/09/2008 – 28/01/2009), and at the Faculty of Life Sciences (LIFE-KU) – University of Copenhagen at the Epidemiology Group of the Department of Large Animal Sciences (02/02/2009 – 04/05/2009).
During all the training period Professor Virgílio da Silva Almeida was the Co-Supervisor.

In the first part of the training period, Dr. Telmo Nunes was assigned as supervisor and the training had as main objective the development of scientific skills in epidemiological & risk analysis and data management under the topic of Bluetongue Surveillance in Portugal.
During the FMV-UTL training period several small projects were developed:

- Project 1:
    - Topic: Bluetongue prevalence estimation in Portugal in 2005 from pre-movement tests data;
    - Learning objectives: bluetongue disease; bluetongue vectors; epidemiological indicators; data management and analysis; data geographical representation;
- Project 2:
    - Topic: Types of confidence intervals for a proportion;
    - Learning objectives: confidence intervals;
- Project 3:
    - Topic: Geographical representation of the evolution of Bluetongue virus restriction zones between 2004 and 2008 in Portugal;
    - Learning objectives: data geographical representation; data management;
- Project 4:
    - Topic: Animal and herd densities in Portugal;
    - Learning objectives: data geographical representation; data management; Kernel densities; sampling processes;
- Project 5:
    - Topic: Risk factors for the *Culicoides* occurrence at herd level in Portugal;
    - Learning objectives: bluetongue risk factors; bluetongue vectors; survey design; data management and analysis; epidemiological measures of association;
- Project 6:
    - Topic: Portugal *Culicoides* occurrence modelling;
    - Learning objectives: bluetongue risk factors; bluetongue vectors; data management and analysis; remote sensing; logistic regression models;

In order to develop the above mentioned projects, the following software programmes were explored: MS Office Access, MS Office Excel, ArcGIS, GvSIG, JMP 7 and SAS.

During the training at FMV-UTL the candidate also participated in:

- Animal Health Department seminars:
    - Presentation:
        - "Types of confidence intervals for a proportion" by Diogo Marques (FMV-UTL) – 21th October 2008, FMV-UTL;
    - Purpose: Overview of the main types of confidence intervals for a proportion used in epidemiology;
    - Participants: Telmo Nunes (FMV-UTL), Hugo Martins (FMV-UTL), Solange Pacheco (FMV-UTL), Ana Duarte (FMV-UTL);

- Workshops:
    - GIS Workshop:
        - Presentation:
            - "Geographical Information Systems (GIS) applied to Epidemiology" by Hugo Martins (FMV-UTL), 13th - 14th October 2008;
        - Purpose: Introduction to GIS, GvSIG and ArcGIS training;
        - Participants: Solange Pacheco (FMV-UTL), Ana Duarte (FMV-UTL), Emanuel Garcia (FMV-UTL), Rui Cepeda (FMV-UTL), Vasco Martins (FMV-UTL), André Silva (FMV-UTL), Miguel Figueiredo (FMV-UTL).

    - Statistics Workshop:
        - Presentation: "Data analysis and statistical tests" by Filipa Matos Baptista (LIFE–KU, FMV-UTL) – 08th - 09th January 2009, FMV-UTL;
        - Purpose: Statistical methods applied to epidemiology, SAS training;
        - Participants: Virgílio Almeida (FMV-UTL), Telmo Nunes (FMV-UTL), Hugo Martins (FMV-UTL), Solange Pacheco (FMV-UTL), Ana Duarte (FMV-UTL), Emanuel Garcia (FMV-UTL).

- Conferences and Congresses:
    - "II Technical Journeys – Caçador Pecuária/Batallé/OPP" - Seminar on Swine Production – 07th November 2008, Leiria;
    - "IV Congress of Veterinary Sciences Portuguese Society / I Iberian Congress of Epidemiology", 27th – 29th November 2008, INRB-INIA/Fonte Boa.

In the second part of the training period the candidate joined the Epidemiology Group working on Paratuberculosis at LIFE-KU – Denmark. Professor Søren Saxmose Nielsen was his supervisor and the main objective of the training was the assessment of MAP occurrence in unprocessed beef and milk.

During the training period two seminars were organized by Søren Saxmose Nielsen:

- Start-up Seminar:
  - Presentations:
    - "Paratuberculosis" by Søren Saxmose Nielsen (LIFE-KU) – 05th February 2009, LIFE-KU;
    - "Short overview of Microbial Risk Assessment" by Sara Monteiro Pires (Food-DTU) - 05th February 2009, LIFE-KU;
  - Purpose: Discuss the objectives, outline, boundaries and expected results of the candidate's work;
  - Participants: Hans Houe (LIFE-KU), Jens Frederik Agger (LIFE-KU), Liza Rosenbaum Nielsen (LIFE-KU), Filipa Matos Baptista (LIFE-KU, FMV-UTL), Lis Alban (Danish Meat Association), Heidi Mikkelsen (Vet-DTU) and Antonio Vieira (Food-DTU).

- Follow-up Seminar:
  - Presentations:
    - "MAP occurrence in unprocessed beef and milk" by Diogo Marques (FMV-UTL) – 28th April 2009, LIFE-KU;
    - "Implication of MAP infection on cattle food chain" by Hisako Okura (LIFE-KU) – 28th April 2009, LIFE-KU;
  - Purpose: Discuss the results of the candidate's work; Provide Hisako Okura inputs to her PhD. study on MAP risk assessment.
  - Participants: Søren Saxmose Nielsen (LIFE-KU), Jens Frederik Agger (LIFE-KU), Liza Rosenbaum Nielsen (LIFE-KU), Nils Toft (LIFE-KU), Erik Rattenborg (Danish Dairy Board), Heidi Mikkelsen (Vet-DTU), Sara Monteiro Pires (Food-DTU), Syed Sayeem Ahmed (LIFE-KU), Torben Dahl Nielsen (LIFE-KU).

ACKNOWLEDGMENTS

At the end of this work, I want to express a word of thanks to all the people who have contributed directly or indirectly to its success:

- To Virgílio Almeida for accepting to be my Co-Supervisor, for his invaluable help and motivation throughout this work and his exciting and always funny Epidemiology teaching;
- To Telmo Nunes for being my unofficial but exceptional Portuguese supervisor, for his constant enthusiastic will of teaching, for encouraging to think on my own;
- To Filipa Matos Baptista for her friendship, motivation, exceptional helping and strength she gave during all training, especially while I was in Denmark;
- To my Portuguese department colleagues Hugo Martins, Ana Duarte and Solange Pacheco for all their pleasant and unforgettable moments spent during the training period;
- To Armando Louzã, Yolanda Vaz, Mário Melo, Maria Isabel Neto, José Pedro Lemos for the funny and always agreeable lunches and "Nespresso Coffee-breaks";
- To Søren Saxmose Nielsen for being my Supervisor. His availability, support and guidance during my stay in Denmark were exceptional and a reference to my future;
- To all my Danish department colleagues for allowing me to be part of the team and enjoy the relaxing and "delicious" moments at the "Monday's Cake Party", especially to Hans Houe for accepting me at the department, to Jeanne Oakman for her constant happiness and contagious laughs, and to Sayeem Ahmed for his company at the office;
- To all the friends I made during my stay in Denmark in particular to Teresa Santos, Ida Engelund, Ana Ortega, Belén Pinilla, Bruno De Meo, Eros Ricci, Agustin, Tamara and Pilar for their friendship and for making my stay in Denmark a great and unforgettable life experience;
- To Sara Pires for the encouragement and inspiration regarding the Epidemiology and Risk Analysis, and for the willingness to help and availability to be present at the seminars;
- To all my other friends Duarte, Anabela, Emanuel, Joana Afonso, Joana Marcelino, Daniela, Tiago, Catarina Antão, Margarida Ramada, Claudia Alves and "BodyAttack-Colleagues" for being always present and for their friendship;
- At last but not the least, I cannot forget to thank to all my family for their support, ongoing motivation and positivism during all good and bad moments. For them a Huge Thank You.

With all my thanks

Diogo Marques

RESUMO

Modelo conceptual da avaliação do risco de ocorrência do *Mycobacterium avium* subspécie *paratuberculosis* em carne e leite de bovino não processados

A etiologia da Doença de Crohn é actualmente desconhecida e o *Mycobacterium avium* subspécie *paratuberculosis* (MAP) tem sido proposto como um dos possíveis agentes etiológicos.

Apesar de não ter sido ainda estabelecida uma relação causal entre a Doença de Crohn e o MAP, este tem sido frequentemente isolado em humanos. O consumo de leite e carne é considerado um possível veículo do MAP, justificando-se por isso a avaliação do risco de ocorrência do MAP nos produtos mencionados.

Este trabalho teve como objectivo avaliar a probabilidade de ocorrência do MAP em carne e leite de bovino, não sujeitos a processamento tecnológico.

De forma a consultar e avaliar informação existente, foi feita uma pesquisa bibliográfica exaustiva sobre a disseminação da infecção por MAP e sobre o isolamento do MAP por cultura bacteriológica de tecidos. A principal lacuna identificada foi a ausência de informação detalhada sobre: i) o mecanismo de disseminação do MAP e a importância da contaminação fecal; ii) a relação entre a disseminação do MAP e outros indicadores (sinais clínicos, lesões macroscópicas, resposta imunitária); iii) a prevalência do MAP na carne e no leite nos diferentes estadios de infecção.

A ausência desta informação não permite a avaliação do risco e a consequente definição de medidas específicas com vista à sua mitigação. Neste trabalho são descritas as árvores de eventos, pressupostos, informação necessária e as lacunas no conhecimento.

Estudos futuros são necessários para disponibilizar a informação inexistente e para desenvolver e aperfeiçoar testes de diagnóstico para detecção directa do MAP na carne e no leite de bovino.

Palavras-chave: *Mycobacterium avium* subspécie *paratuberculosis*; Infecção disseminada do MAP; Leite; Carne; Bovinos; Árvore de eventos; Análise de risco.

ABSTRACT

Risk assessment conceptual model of occurrence of *Mycobacterium avium* subspecies *paratuberculosis* in unprocessed beef and bovine milk

Crohn's Disease aetiology is currently unknown and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has been proposed as its etiologic agent.

Despite the absence of a causal relationship between MAP and Crohn's Disease, MAP has been frequently isolated in humans. Milk and beef consumption is considered a possible MAP source. Thus the risk of MAP occurrence on these products should be assessed.

The main objective of this work was to assess the probability of MAP occurrence in unprocessed beef and bovine milk.

In order to assemble the available data, an exhaustive literature search was made on the dissemination of MAP infection and MAP isolation by bacteriologic tissue culture. The main knowledge gaps found were the lack of detailed information on: i) MAP dissemination mechanism and the importance of faecal contamination; ii) relation between MAP dissemination and other indicators (clinical signs, gross lesions, immune response); iii) MAP prevalence on beef and milk by stage of infection.

Due to the lack of this information, the risk assessment and the characterization of the risk mitigation measures could not be performed. The risk model pathways, its assumptions, data required and knowledge gaps are described in this work.

Further research is needed to make available the mentioned knowledge gaps and to develop and to improve diagnostic tests for direct MAP detection on beef and bovine milk.

Keywords: *Mycobacterium avium* subspecies *paratuberculosis*; Disseminated MAP infection; Milk; Beef; Bovine; Risk model pathway; Risk assessment.

CONTENTS

TABLE LIST

FIGURE LIST

EQUATION LIST

ABBREVIATIONS LIST

AGID	Agarose Gel Immunodiffusion
CD	Crohn's Disease
CF	Complement Fixation
ELISA	Enzyme-Linked Immunosorbent Assay
IFN-γ	Interferon gamma
LN	Lymph Node
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
OIE	Office International des Epizooties - The World Organisation for Animal Health
ParaTB	Paratuberculosis
PCR	Polymerase Chain Reaction
Se	Sensitivity
Sp	Specificity
ZN	Ziehl-Neelsen

1 Introduction

1.1 Project objectives

During 3 months, the candidate joined the Epidemiology Group of the Department of Large Animal Sciences at the Faculty of Life Sciences – University of Copenhagen. This was possible due to a student mobility grant of ERASMUS/SOCRATES programme.
In the framework of the Danish control programme of bovine paratuberculosis, a start-up seminar of MAP occurrence on beef and milk was organized and experts on relevant matters were present. During the seminar, the objectives, outline, boundaries and expected results of the present dissertation were discussed.

The main objective initially proposed was the assessment of MAP occurrence in unprocessed beef and bovine milk. In order to accomplish this objective, an exhaustive literature-based search was made to understand the relevant aspects of Paratuberculosis and MAP infection, to assess the current knowledge and to identify unavailable inputs required to develop the proposed risk assessment.

Due to considerable knowledge gaps it was not possible to accomplish the main objective but the available and non-available risk assessment inputs were described and proposals for MAP risk assessment model pathways were made.

This dissertation is composed by four parts:

1. Introduction (objectives and problem definition);
2. Overview of Paratuberculosis and MAP infection;
3. Literature revision concerning MAP isolation by tissue culture and MAP distribution on tissues;
4. A proposal of risk assessment model pathways to assess the risk of MAP occurrence in unprocessed beef and bovine milk.

1.2 Problem definition

1.2.1 *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's Disease

Crohn's Disease (CD) is a chronic disease that affects the gastro-intestinal tract of humans. Usually, it occurs in young adults showing as major symptoms diarrhoea and chronic pain (Grant, 2005; Waddell et al., 2008).

Chiodini, Van Kruiningen, Merkal, Thayer & Coutu (1984) isolated *Mycobacterium* sp. organisms from several patients with CD. This finding revived the idea of a mycobacterial cause for this human disease (Chiodini, Van Kruiningen, Merkal, Thayer & Coutu, 1984). Since then, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has been isolated from several patients and a possible link between CD and MAP has been discussed in the last decades. Some of the findings that support this link are: the similarities of clinical signs between CD and bovine paratuberculosis (in which MAP is the etiological agent); detection of MAP on faeces, intestinal tissues and blood from CD patients; demonstration of a serological response to MAP antigens in Crohn's patients; and anti-MAP antibiotic therapy resulting in remission or reduction of clinical signs. Several potential roles of MAP in CD patients have been suggested: MAP could be the primary infectious agent of CD; a secondary invader in a host with a compromised intestinal barrier; or colonize the intestinal tract without causing any harm (Grant, 2005). Because there are inconclusive and contradictory studies, difficulties on the isolation of MAP organisms and unexpected results, it is very difficult to achieve a consensus between authors (Waddell et al., 2008; Grant, 2005). In a recent systematic review, Waddell et al (2008) analyzed many studies and several MAP isolation methods in CD patients and concluded that the "evidence of association is not strong, but should not be ignored". Despite the absence of an irrefutable causal relationship between MAP and CD, MAP has been consistently isolated in humans and its source should be investigated. Food and water-borne sources have been suggested (Grant, 2005).

1.2.2 Human MAP sources - Water

Water as a MAP source has not been properly investigated and its potential as a vehicle of transmission of MAP to humans is unknown (reviewed in: Grant, 2005)[1]. Water containing MAP may be consumed directly, incorporated into food, used to wash food products or surfaces used to manipulate food. MAP survival characteristics in water environments were analyzed by Larsen, Merkal & Vardaman (1956), Lovell, Levi & Francis (1944) and Whittington, Marshall, Nicholls, Marsh & Reddacliff (2004). The presence of mycobacteria in

[1] The connotation "reviewed in" is used to differentiate review from original articles.

piped supplies suggests that water standard treatments have little effect on these organisms. The chlorination effect was studied in experimentally contaminated water and MAP was not completely eliminated by this process (Whan, Grant, Ball, Scott & Rowe, 2001). These findings make water a plausible MAP source that should be properly investigated.

1.2.3 Human MAP sources – Beef and Milk

Beef, milk and milk products are food products of bovine origin largely consumed by humans. There are no specific regulations about MAP presence on food products. Thus contaminated milk and beef may be consumed by humans.

MAP occurrence on milk and milk products was studied by several authors and through different methods (reviewed in: Slana, Paolicchi, Janstova, Navratilova & Pavlik, 2008). MAP was isolated by culture on milk samples aseptically collected from individual cows (Alexejeff-Goloff, 1929; Streeter, Hoffsis, Bechnielsen, Shulaw & Rings, 1995; Sweeney, Whitlock & Rosenberger, 1992a; Taylor, Wilks & McQueen, 1981; Paolicchi et al., 2003; Ayele, Svastova, Roubal, Bartos & Pavlik, 2005; Pillai & Jayarao, 2002; Giese & Ahrens, 2000; Jayarao et al., 2004). MAP was also identified on milk and milk products after industrial processing steps and their effect on MAP survival or mitigation was discussed (Slana et al., 2008; reviewed in: Grant, 2006). However, MAP resistance to milk industrial processing steps is not broadly accepted due to contradictory results and some study designs were criticized. Differences between the laboratory techniques used and industrial procedures are the reasons suggested for these differences, e.g. initial MAP concentrations; MAP origin - culture collection/field strains; absence/presence of turbulent flow during heating to assure that all milk particles get the same treatment; heating methods, volumes of milk tested; and absence/presence of homogenization step (reviewed in: Grant, 2006; Rademaker, Vissers & Giffel, 2007).

Presence of MAP after various processing procedures is not fully understood. It may be attributable to its high heat resistance (e.g. cell clumping, phagocytosis) (Rademaker et al., 2007) or to contamination during the process. It can be due to cross contamination during laboratory analysis; leaks on the heat exchanger plates of pasteurization plants enabling raw milk to contaminate pasteurized milk; and use of pasteurizers during a long time between cleaning processes (milk dry matter deposits leading to decrease in heat treatment efficacy) (Cerf, Griffiths & Aziza, 2007).

Map isolation and quantification constrains are also encountered in the evaluation of milk processing steps. The real number of MAP present in raw or pasteurized milk cannot be precisely estimated because milk culture requires a chemical decontamination treatment which has adverse effects on MAP viability (Dundee, Grant, Ball & Rowe, 2001; Grant &

Rowe, 2004) ending on an underestimation of MAP numbers (Grant, Williams, Rowe & Muir, 2005). The dilution on bulk milk tank also influences the detection of MAP on raw and processed milk. Despite the lack of consensus on the effect of milk processing steps on MAP, the evidence of MAP occurrence on retailed milk makes this product a key MAP source to be investigated.

Regarding beef, little information is available about this possible source of MAP to consumers. MAP was isolated by culture from muscle tissue (Antognoli et al., 2008; Alonso-Hearn et al., 2009), MAP DNA was obtained from surface swabs collected from beef carcasses at the slaughterhouse after skinning and dressing (Meadus, Gill, Duff, Badoni & Saucier, 2008), and two MAP detection methods in ground beef were compared (Jaravata, Smith, Rensen, Ruzante & Cullor, 2007).
MAP organisms may be reduced or eliminated by heating during cooking procedures. However, the effect of heating processes on MAP survival in the muscle matrix has not been evaluated (Alonso-Hearn et al., 2009). Raw and undercooked meat consumption should also be considered. Some products such as sausages and minced meat are based on low quality meat, resulting in a larger probability of coming from infected cattle with high MAP contamination rates (Alonso-Hearn et al., 2009). Beef as MAP food source to consumers should be subjected to further research.

1.2.4 Map occurrence on products entering the food chain and the support of the risk assessment

Considering these three MAP sources to humans (water, milk and beef), the reason behind MAP presence on these products should be assessed.
Water contamination has been mainly attributed to contamination with faeces of infected animals and to MAP capacity to survive for long periods of time on the environment (reviewed in: Grant, 2005).
MAP isolation from milk, muscle and other tissues, for example non gastro-intestinal lymph nodes and internal organs led some authors to suggest the possibility of MAP dissemination inside the infected host (Alonso-Hearn et al., 2009; Antognoli et al., 2008; Dennis et al., 2008; Hines, Buergelt, Wilson & Bliss, 1987; Pavlik et al., 2000). Yet there is also the chance of contamination by MAP of faecal origin during tissue collection and/or food processing.
The view of MAP entry into the food chain, its potential to survive along food processing, the lack of MAP regulations, the increased claim of food safety by consumers, the continuous search for better and more competitive products by the dairy and meat industries, and the commitment to promote Public Health by the veterinary authorities, made this mycobacteria a subject of study. In order to properly address the MAP source to humans, a MAP risk

assessment on the food chain would be of great importance for risk managers to take better and more cost-effective decisions.

This work will therefore contribute to the development of such assessment in the most important products of animal origin, beef and milk. Water as a MAP source was excluded regarding the amount of knowledge gaps and the lack of published studies. Therefore, and due to time limitations, animal products consumed by humans were given priority.

The assessment of MAP occurrence in beef and milk will be addressed as a qualitative risk assessment and will be limited to a release assessment, i.e. the final objective will be to evaluate the probability and the quantity of MAP shed by infected animals into specific animal products.

The exposure assessment step was not incorporated in this study because:

- the causal relationship between MAP and Crohn's disease has not been established;
- MAP doses that causes an undesirable effect on humans are unknown;
- there is a lack of consensus about the effect of food processing steps on MAP survival/reduction.

As this dissertation was elaborated in the framework of a curricular training period in Denmark, the Danish control programme of bovine paratuberculosis will be used as a model.

The assessment will be restricted to cattle because of the larger amount of scientific publications and the special focus on cattle of the Danish bovine paratuberculosis control programme.

The end-points assumed will be the milk at herd level (individual milk) and the carcass at the end of the slaughter line. These end-points are in agreement with the purpose of the release assessment.

This work will focus on MAP dissemination inside the host, rather than on faecal contamination during harvesting and processing steps.

2 Paratuberculosis

2.1 Aetiology

Paratuberculosis (ParaTB) is a chronic, infectious disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It is a Gram-positive, intracellular facultative, acid-fast bacillus that is slow-growing, and has complex nutritional requirements in culture. It requires an organic source of iron and mycobactins to grow *in vitro* (reviewed in: Chiodini, Van Kruiningen & Merkal, 1984). Through genomic DNA analysis it was shown that it has an unique insertion sequence: IS*900* (Green et al., 1989). The genus *Mycobacterium* consists of about 50 species that are grouped according some similarities. The most known are the *Mycobacterium tuberculosis* complex (including *M. tuberculosis* and *M. bovis*), *Mycobacterium leprae* and *Mycobacterium avium-intracelullare* complex (including *Mycobacterium avium* and MAP) (Buergelt, Bastianello & Michel, 2004; Coetzer & Tustin, 2004). Cell wall elements of mycobacteria not only help in the survival and resistance of the agent against host defences but are also responsible for the host strong immunological response (reviewed in: Clarke, 1997). MAP has been shown to survive on the environment at different conditions. It can survive not only in dry conditions (Larsen, Merkal & Vardaman, 1956) but also in freezing environment (Larsen et al., 1956; Richards & Thoen, 1977) or water, soil and faeces (Jorgensen, 1977; Larsen et al., 1956; Lovell, Levi & Francis, 1944; Whittington, Marshall, Nicholls, Marsh & Reddacliff, 2004). It may stay viable in slurry, even when submitted to heat treatments (Olsen, Jorgensen & Nansen, 1985; Jorgensen, 1977) and in different water sources (Whittington, Marsh & Reddacliff, 2005). Recent studies suggest rather effective survival strategies namely an interaction between MAP, protozoa, nematodes and insects (Rowe & Grant, 2006) and the move into a dormancy state when MAP is submitted to unfavourable conditions (Whittington et al., 2004).

2.2 Epidemiology

Paratuberculosis occurs on several ruminant species but it is most studied on domestic ruminants. It is a widespread infection in Europe with cattle within-herd prevalence ≈ 20% and between-herd > 50% in cattle herds and > 20% in small ruminants flocks (Nielsen & Toft, 2009).

MAP can be transmitted by either direct or indirect contact between infected and susceptible animals. Transmission occurs mainly by faecal-oral route. Shedding animals play an important role contaminating the feed, water and pastures (reviewed in: Clarke, 1997). Transmission can also occur *in utero* (Doyle, 1958; Sweeney, Whitlock & Rosenberger, 1992b), by ingestion of infected colostrum and milk from infected cows or when the teats are

contaminated with faeces (Streeter et al., 1995; Sweeney et al., 1992a). Contaminated feeding utensils, herd worker's shoes or clothing, wildlife ruminant reservoirs and certain veterinary procedures (e.g. rectal examination) can also transmit MAP (Sweeney, 1996). Between-herd transmission is thought to be mostly caused by purchases of healthy carriers. However, a break on the herd biosecurity or a direct contamination between herds, for example a common water source, may result on a cluster of paratuberculosis cases bearing in mind the resistance of MAP in environment (Sweeney, 1996; Whittington et al., 2005).

The animals that are more vulnerable to infection are the neonates and some studies point to the existence of an age-dependent resistance (Bendixen, 1978; Buergelt, Hall, McEntee & Duncan, 1978; Larsen, Merkal & Cutlip, 1975; Rankin, 1958; Rankin, 1961b; Rankin, 1961a; Rankin, 1962; reviewed in: Windsor & Whittington, 2009). Hypothesis to explain calves' susceptibility are: (i) an age-related difference on cellular immune response (Windsor & Whittington, 2009); (ii) effect (dilution or harmful effect) that the functional rumen of adult animals may have on MAP before they reach the gut (Windsor & Whittington, 2009); (iii) the existence of an "open gut" in calves that besides allowing some macromolecules such as immunoglobulins from colostrum to penetrate the mucosa to be absorbed, it may permit the entrance of MAP into the animal intestinal wall (Sweeney, 1996). Some animals could become asymptomatic carriers and shed the agent during their lives (reviewed in: Chiodini et al., 1984). Passive transfer or a pass-through of MAP is referred when a faecal culture positive exist together with a tissue culture negative (however the sample collection procedure may have neglected the infected sites) (Whitlock, Rosenberger, Sweeney & Spencer, 1996). Passive shedding has also been reported after oral inoculation and from animal living in a heavily contaminated environment (Hines II et al., 2007; Sweeney, Whitlock, Hamir, Rosenberger & Herr, 1992)

2.3 Pathogenesis and Clinical signs

The pathogenesis of Paratuberculosis can be divided into three main stages of infection: early infection, subclinical infection and clinical disease (Coussens, 2001).

MAP is ingested orally with contaminated material. Mycobacteria are transported in vacuoles across the M cells to macrophages in Gut Associated Lymphoid Tissue (mainly in Peyer's patches) (Momotani, Whipple, Thiermann & Cheville, 1988). These patches reach their maximum development about the time of birth and progressively disappear afterwards (reviewed in: Clarke, 1997; Reynolds & Morris, 1983). After undergoing phagocytosis MAP organisms suffer degradation activities within the macrophage, are processed and presented to T lymphocytes or remain intact inside the phagocytic cell (Coussens, 2001; Stabel, 2000b). During the subclinical stage of infection there are no clinical symptoms. A granuloma develops that may allow the growth of the pathogen inside it by protecting infected cells from

cytotoxic immune effects (reviewed in: Coussens, 2001; Zurbrick & Czuprynski, 1987). Sporadic destruction of infected macrophages inside granulomas can lead to bacterial shedding observed in faecal cultures from subclinically infected animals. Intermittent shedding not only provides a way to MAP moving to new infection sites, leading to large damage portions of the intestinal tract but also to a continuous low-level stimulation of humoral immunity. This could convert a low-undetectable antibody response into detectable levels of antibodies in middle to late-stage of subclinical infections (reviewed in: Coussens, 2001). The initial cell-mediated response of the early and subclinical stages of infection is replaced by a non-protective humoral response at the clinical stage (Coussens, 2001; Stabel, 2000b; Stabel, 2000a) allowing for a rapid dissemination of the infection throughout the host (Bendixen, 1978; Stabel, 2000a) and the development of new lesions.

The balance between host (local and systemic immunity, age), MAP (infective dose, route of infection, virulence and survival capabilities) and environmental factors will determine the progression from the subclinical stage to the appearance of symptoms.

The symptoms are usually unspecific like a chronic progressive weight loss with chronic or intermittent diarrhoea. However, in advanced stages of infection, animals may show mandibular oedema, hypoproteinaemia and become cachetic or even die (reviewed in: Clarke, 1997). Correlation between disease progression and some measurable production indicators such as milk yield has also been investigated. Although is not an observable clinical sign, deviations in milk yield can be used together with other laboratory diagnosis techniques to characterize the stage of infection and the animal status, and take management measures (Nielsen, Krogh & Enevoldsen, 2009; Nielsen & Toft, 2008). Though young animals are the more susceptible to infection, clinical disease generally develops only after 3-5 years of age (Chiodini et al., 1984). Animals with clinical signs are considered the "tip of the iceberg" (Whitlock & Buergelt, 1996)[2]. However infected animals have higher probability of being culled due to secondary infections, such as mastitis and metritis, than from severe clinical stages of late disease (Merkal, Larsen & Booth, 1975).

2.4 Pathology

Usually the concomitant existence of clinical symptoms, pathological signs and isolation of organism are representative of a later stage of the disease in contrast to early stages where this association is not a routine finding (Brady, O'Grady, O'Meara, Egan & Bassett, 2008; Clarke, 1997; Pavlik et al., 2000; Pérez, Marín & Badiola, 1996).

In a study of 32 infected cattle by Buergelt et al (1978) the most common pathologic gross findings were chronic enteritis, chronic intestinal lymphangitis and mesenteric

[2] Whitlock & Buergelt (1996) suggested that for each 3 animals with clinical signs (stages III and IV) may exist 8 animals with subclinical disease (stage II) and 14 animals with silent infection (stage I) in an population with 25 infected animals.

lymphadenopathy with major lesions present at the distal ileum. Other pathological signs observed were corrugated and thickened intestinal mucosa, prominent and dilated subserosal lymphatics, cachexia, or atrophy of skeletal muscle and fat. Less frequent lesions were alopecia, and endocardial and aortic calcification. Gross lesions were detected rarely in colon, cecum or rectum (Buergelt et al., 1978).

Various histopathological lesions types can be observed both in sheep (Carrigan & Seaman, 1990; Clarke & Little, 1996; Pérez et al., 1996; Rajya & Singh, 1961; Stamp & Watt, 1954) and cattle (Buergelt et al., 1978; González et al., 2005). Some theories to explain these different lesions types are: (i) that the pathogenicity may vary between MAP strains (Stamp & Watt, 1954); (ii) that this variety of lesions is dependent upon the host immune defences (Bendixen, 1978; Buergelt et al., 1978; Shulaw, Bechnielsen, Rings, Getzy & Woodruff, 1993; Stamp & Watt, 1954); (iii) or that different lesions represent different stages of the disease (Pérez et al., 1996; Rajya & Singh, 1961). In cattle, the histopathologic lesions were described by Buerguelt et al (1978) and by González et al (2005). The latter examined 167 animals and in 116 (70%) they found MAP infection associated lesions which were categorized on focal, multifocal, diffuse multibacillary, diffuse lymphocytic and diffuse intermediate. Focal lesions were observed in distal ileum and lymph nodes and were described as “well-demarcated, small granulomas formed by macrophages with abundant, slightly foamy, pale cytoplasm and large nuclei with sparse chromatin”. On the other hand multifocal lesions (the correspondent to the “mild” and “moderate” forms described by Buerguelt et al (1978)) correspond to focal and well-demarcated granulomas in intestinal lamina propria in addition to lymphoid tissue. It was also observed that sometimes the same animal showed lesions in different regions of the intestine. In diffuse lesions (the correspondent to the “advanced” lesions described by Buerguelt et al (Buergelt et al., 1978)) an inflammatory infiltrate occurred in several areas of the intestine causing diffuse and severe granulomatous enteritis. Animals were divided into three different subtypes according to the main cell type present in the infiltrate and the amount of acid-fast bacteria (AFB). Diffuse multibacillary lesions were named when “macrophages, with foamy cytoplasm and also the appearance of epithelioid cells” appeared diffusely infiltrated in the intestinal wall and mycobacteria were present in large numbers in all sections of the intestine and lymph nodes. Another type of diffuse lesion is the lymphocytic or paucibacillary type where “lymphocytes were the main inflammatory cells infiltrating the lamina propria”. Although AFB was present, they were always in low numbers. González et al (2005) described also an intermediate diffuse category in which the “infiltrate contained abundant lymphocytes, plasma cells, giant cells and macrophages, either isolated or forming small granulomas”. However there was variation in the cellular content of the infiltrate between animals and between samples from the same animal. In this type of lesion mycobacteria were always detected (González et al., 2005).

Though it is uncertain whether different pathological forms, especially diffuse lesions, represent sequential or divergent stages of paratuberculosis, an association between histopathologic lesions and immunity responses was transposed from human leprosy to ruminant paratuberculosis (mainly in sheep) (González et al., 2005). According to that multibacillary lesions may be associated with marked humoral peripheral responses, paucibacillary lesions with a strong cellular immune response, and focal lesions with initial or latent forms of infection associated with high cellular immune responses (Clarke & Little, 1996; González et al., 2005; Pérez et al., 1996).

2.5 Danish Paratuberculosis Control Programme

(adapted from Nielsen (2007) and Nielsen, Jepsen & Aagaard (2007))

The Danish control programme for paratuberculosis started in February 2006. This voluntary programme aims to provide farmers with tools to control paratuberculosis and to reduce the prevalence of this disease. The programme follows a risk-based approach at farm level, where the cows are classified to different groups representing different stages of infection in order to manage the risk according to each risk group.

By June 2007 approximately 1140 (23%) dairy herds of the 4900 Danish dairy herds joined the programme. These herds are primarily large herds with an average herd size of 137 cow/herd compared to the national average of 110 cows/herd (Nielsen, 2007). The farmer's main purposes of participation in the programme were the certification with 4-10 years, the control to avoid production losses, the control to increase animal health and control to increase food safety (S.S. Nielsen, personal communication, February 5th, 2009).

2.5.1 Diagnostic testing

The distribution of animals by risk groups is done according to the result of an individual antibody ELISA test performed on milk quality control samples without an obligatory confirmatory test (faecal culture). An ELISA positive test result is considered when corrected optical density values are > 0.3.

Each herd is tested 4 times a year. Each cow is tested 3 times year (not tested during the dry period). This testing scheme and the test used have as objective an early detection of the infectious animals being the most sensitive as possible. Due to the high test frequency an overall low specificity of the combined test is expected.

The test gives many positive results due to the focus on sensitivity rather than specificity. This characteristics needs to be taken into account in policy making. Every test-positive animal is regarded as a possible MAP disseminator, however, there are several types of status regarding the test positive results: animals could be infectious; could be infected but

still have to progress to disease; could acquire MAP sporadically from a contaminated environment; some could be false-positive.

It is recommended that cows aged 2 – 4 years should be tested more frequently and older cows may be tested less frequently (Nielsen & Ersboll, 2006). It was shown that ELISA test is able to detect almost all infected cattle that shed MAP (Nielsen & Ersboll, 2006), although the age-span of testing positive is from 2 to 11 years of age. With frequent testing most animals are detected at the time they are infectious (Nielsen & Toft, 2006).

2.5.2 Risk levels

Regarding the three colour system for risk groups, the test negative animals are referred as "Green cows". These cows, on the day of the test, are considered non-infectious, possibly not infected and are considered low risk cows. Cows with the classification Yellow (intermittent positive and negative results) and Red (frequent positive results) are infected, infectious and considered high-risk cows. The Yellow cows could be in a stage of controlling the infection and they may be clinically affected. Red cows are in a stage incapable of controlling the infection and are all considered affected.

Another classification by "infection group" is used to infer differences on milk production losses. Based on repeated testing, cows are divided into 6 infection groups regarding their antibody profiles (Nielsen, 2007; Nielsen, 2008) (Fig. 1):

- A0 – repeated negative (minimum 2 samples);
- A1 – negative, but only 1 test result (usually at the beginning of the 1st lactation);
- A2 – positive on last sample and negative on previous test;
- A3 – last 3 tests negative, but with 1 previous positive result;
- A4 – last result negative, but more positive results have occurred previously (fluctuating response);
- A5 – last 2 or more results positive;

Cows with positive reactions are considered to be high risk animals and potentially infectious but only cows from groups A4 and A5 have reduced milk production[3]. The allocation of these animals in the animal status classification is:

- Infected – A2, A3, A4, A5;
- Infectious – A2, A3, A4, A5 – but most important are A4 and A5 (milk yield decreased);
- Affected –A4 and A5.

[3] A decline in milk production is associated with progression of MAP infection and antibody profiles. It was assessed that milk production losses can start 300 days before the first positive antibody test (Nielsen et al., 2009). This information is important to characterize the animal status and to evaluate the stage of infection.

2.5.3 Recommendations

Selective culling strategies should be based on the following conditions: repeated high test-levels; affected animal (with decreased milk yield, diarrhoea, high SCC and other clinical signs), within-herd prevalence of Red and Yellow cows; other factors such as lameness, age and poor performance in general. Recommendations by the three colour risk groups are:

- Green older cows are ideal for colostrum production. Green cows may calve other Green cows. The hygiene level can be lower than high risk cows;
- Red cows are recommended not to calve again. Should be culled prior the next calving and not allowed near the calving area;
- Yellow cows may calve again under special conditions. They should calve in an isolation of Green cows, should be kept in a single pen and thoroughly cleaned after each calving;
- Yellow and Red cows should not provide milk or colostrum to feed calves.

2.5.4 Herd classification

The probability of freedom of MAP infection among cows in a herd is estimated based on annual ELISA testing without obligatory confirmatory testing. This probability is calculated based on test prevalence and its correction for test imperfection (True Prevalence).

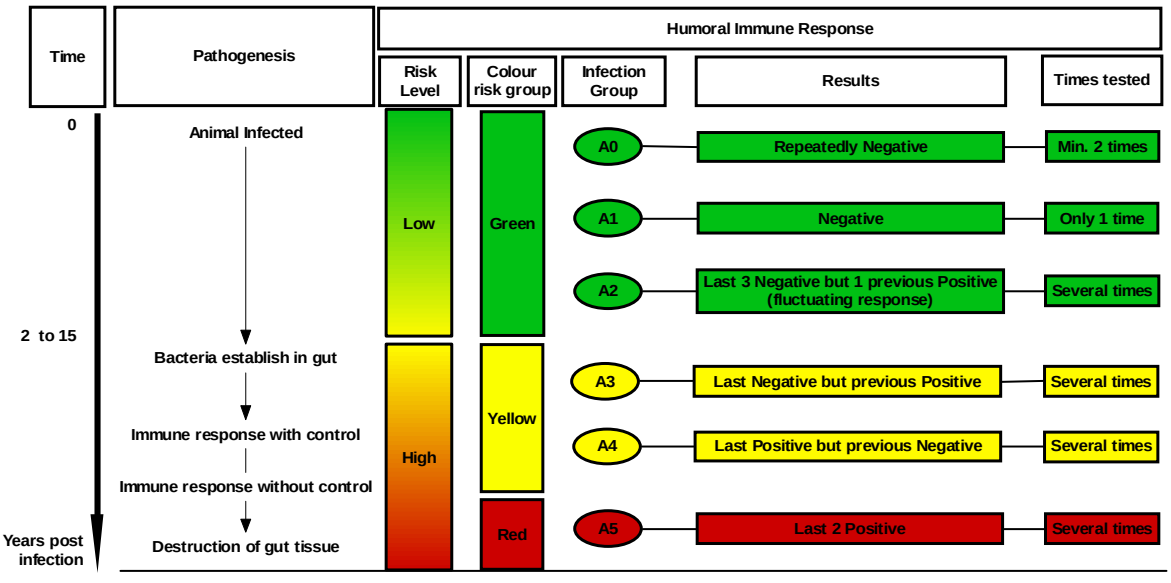

Fig. 1 – Schematic representation of the different risk levels and risk groups from the Danish ParaTB control Programme regarding the pathogenesis of MAP infection (adapted from Nielsen & Toft (2008)).

2.6 Animal status and Stages of Infection

In the context of this dissertation the stages of infection and the animal status will be described.

2.6.1 Stages of infection

General nomenclature

Inapparent (silent) infection[4]:
Is the absence of observable clinical signs and of changes in measurable production indicators (Thrusfield, 2005).

Subclinical disease:
Is defined when "infection occurs without overt clinical signs" but may include changed measurable production indicators (Thrusfield, 2005) as reduction in milk yield and chronic weight loss.

Clinical disease:
This denomination is used when cattle express typical clinical signs of the disease and changes in measurable production indicators.

Affected animals:
Animals that may have production indicators decreased, such as milk yield or animal weight, and/or observable clinical signs of MAP infection;

[4] In the context of this dissertation these animals are also considered as "asymptomatic animals". "Asymptomatic" nomenclature is used on the literature revision regarding MAP distribution in tissues, in opposition to "affected" animals because it is broadly used in published studies.

Stages of Infection description

Three stages of infection were proposed by Coussens (2001) to explain the relation between MAP infection, host immunity response and existence of clinical signs. Other authors proposed a four stage disease description (adapted from: Whitlock & Buergelt, 1996; Whitlock, Wells, Sweeney & Van Tiem, 2000) (Fig. 2):

- Stage I – Silent / Inapparent Infection:
    - Age group: Calves, heifers, young livestock up to 2 years;
    - Clinical signs: No;
    - Measurable production indicators (weight and milk yield): Normal;
    - Detectable antibody response: No;
    - Detectable MAP shed in faeces: No;
    - MAP demonstration in tissues: Yes;
    - Possible animal status: Infected.

- Stage II – Subclinical disease:
    - Age group: Adults;
    - Clinical signs: No;
    - Measurable production indicators (weight and milk yield): Decreased;
    - Detectable antibody response: Yes;
    - Detectable MAP shed in faeces: Intermittently;
    - MAP demonstration in tissues: Yes;
    - Possible animal status: Infected, probably infectious and affected.

- Stage III and IV – Clinical disease and Advanced clinical disease:
    - Age group: Adults;
    - Clinical signs: Yes;
    - Measurable production indicators (weight and milk yield): Decreased;
    - Detectable antibody response: Yes;
    - Detectable MAP shed in faeces: Yes;
    - MAP demonstration in tissues: Yes;
    - Possible animal status: Infected, infectious and affected.

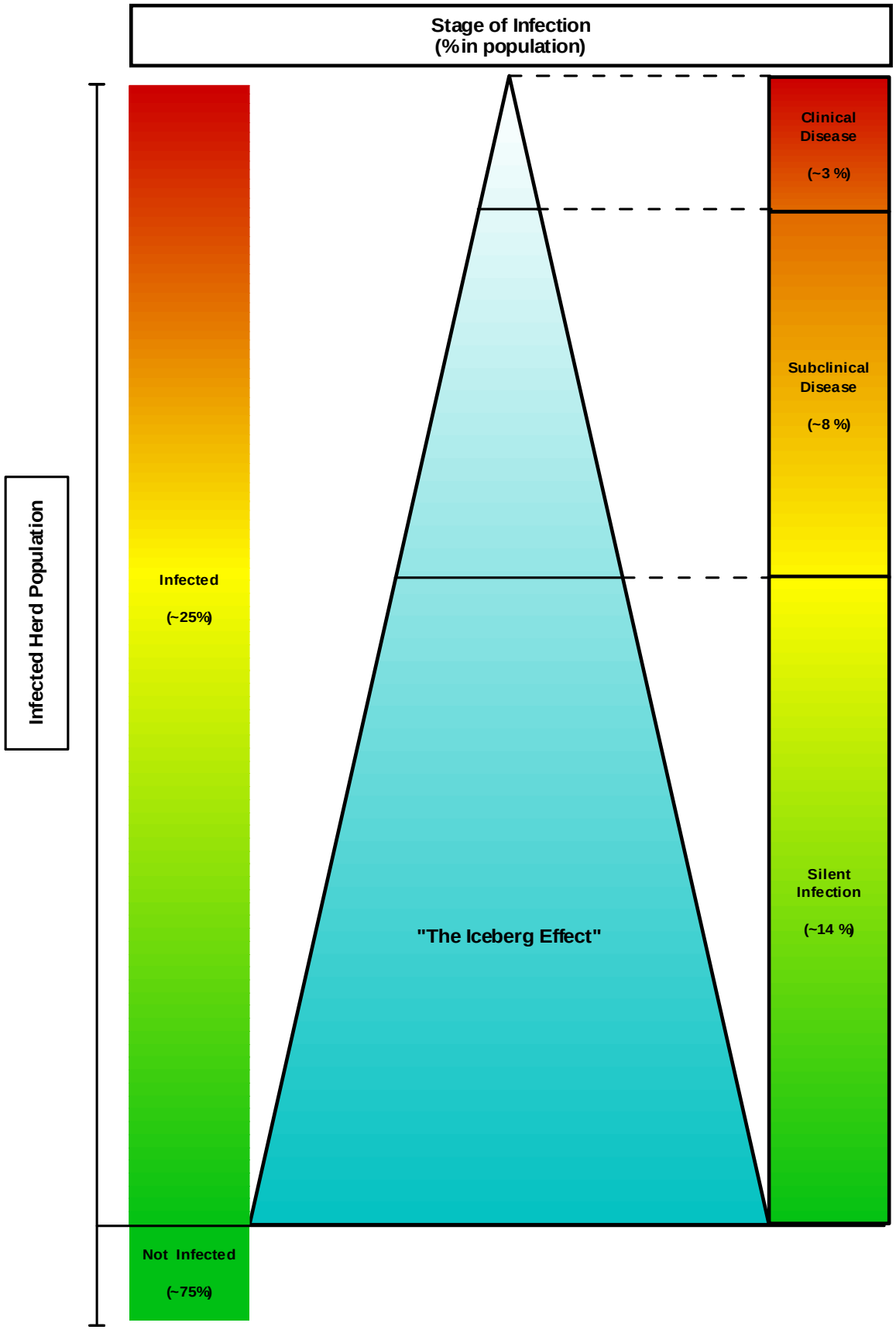

Fig. 2 – Schematic representation of “The Iceberg Effect” and the Stages of Infection in an infected herd. Proportions adapted from Whitlock & Buergelt (1996) considering a population of 100 animals with 25 infected.

2.6.2 Animal Status

The following animal status classification was proposed on an evaluation study of the diagnostic tests accuracy and will be adopted in this dissertation. Animals were classified in Infected, Infectious and Affected (adapted from: Nielsen & Toft, 2008) (Fig. 3):

- Infected animals:
    - In these animals MAP persistence lasted long enough to stimulate an immune response, to produce clinical signs or to be detected by agent cultivation;
    - Cattle assigned to this status may become infectious and affected in later stages of infection;
    - It is assumed that once a cow has an established infection, the infection persists for life;
    - These animals pose a risk of becoming infectious and cannot be declared MAP-free;
    - They are an economic burden when there is decrease in milk/weight and transmission to other animals.

- Infectious animals:
    - These animals shed MAP at the time of testing according to the test used;
    - Their infectious status can also be decided on the basis of the detection of an immune response (Nielsen, Grohn & Enevoldsen, 2002);
    - These animals are considered infected and may be affected;
    - They may include non-infected animals which may be passive shedders of MAP (transient infectious status);
    - MAP shedding is mainly observed in faeces, but it may also occur in milk and be transferred *in utero*.
    - These animals are considered an economic burden because in short-term they experience decreased milk production and on long-term they transmit MAP to susceptible animals in the herd.

- Affected animals:
    - This status is assigned when cattle have decreased milk yield or weight loss (or any other production indicator) and/or clinical signs characteristic of MAP infection;
    - These animals are considered infected and probably infectious;
    - Their value is low because they may have reduced milk yield, decreased weight and probably low value at slaughter. There is a risk they will die from the infection.

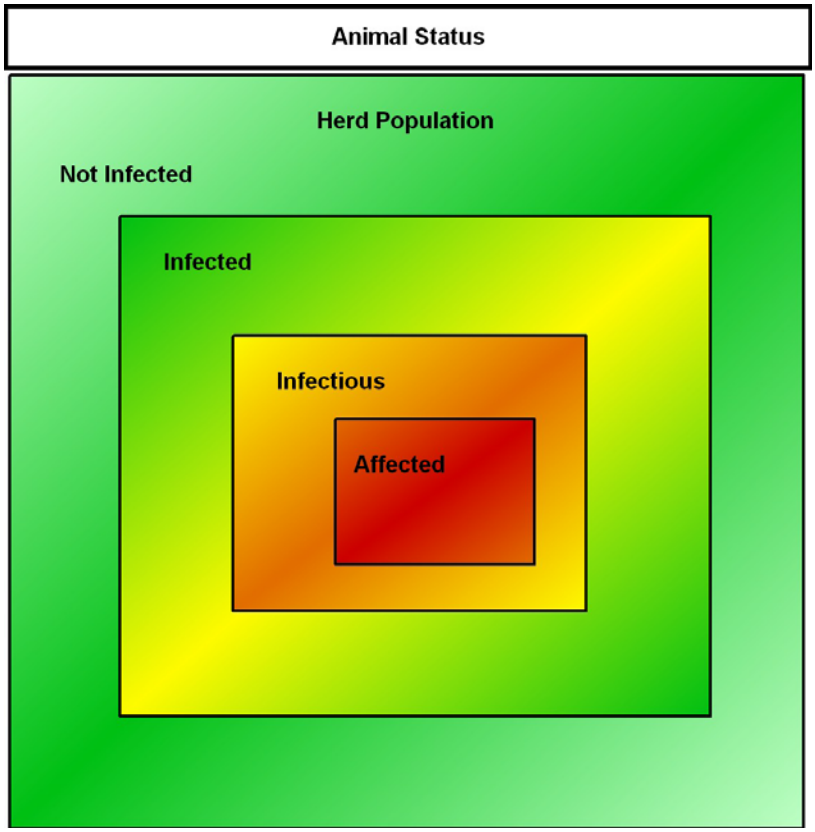

Fig. 3 – Schematic representation of the different animal status in an infected herd population. Proportions for each status are unknown.

2.7 MAP diagnostic methods

None of the available diagnostic methods are 100% MAP specific and sensitive. Thus, detection and characterization of MAP infections are a hard task. MAP infection may be detected by clinical diagnosis or laboratory techniques. Due to the dynamics of MAP infection both options are dependent on the stage of infection and animal status.

MAP clinical signs were described previously. Usually they are associated with advanced stages of infection and must be confirmed by laboratory techniques. MAP suspicion by clinical diagnosis is influenced by veterinarian diagnostic skills, herd owner disease knowledge and capacity to detect affected animals, and the type of clinical signs. Clinical diagnosis sensitivity and specificity are unknown. In some countries, due to specific MAP legislation, the fear of being officially classified as MAP infected herd results in underreporting of suspicious signs. This fact decreases the sensitivity of the clinical diagnosis (Anon, 2004b).

A laboratory diagnostic test should be chosen based on the analyte to be detected (bacteria, bacterial constituents, immune response). Due to dynamics of the MAP infection the immune response, bacterial load, potential shedding or MAP dissemination through the host do not necessarily follow the same pattern or time course. Therefore, the major obstacle for obtaining a correct diagnosis is the time-dependent responses (Nielsen, 2002).

Two main diagnostic patterns can be followed: techniques that detect the agent and techniques that detect the immune response. MAP laboratory diagnosis methods were described elsewhere (Nielsen, Nielsen, Huda, Condron & Collins, 2001) (Fig. 4).

2.7.1 Diagnostic test types

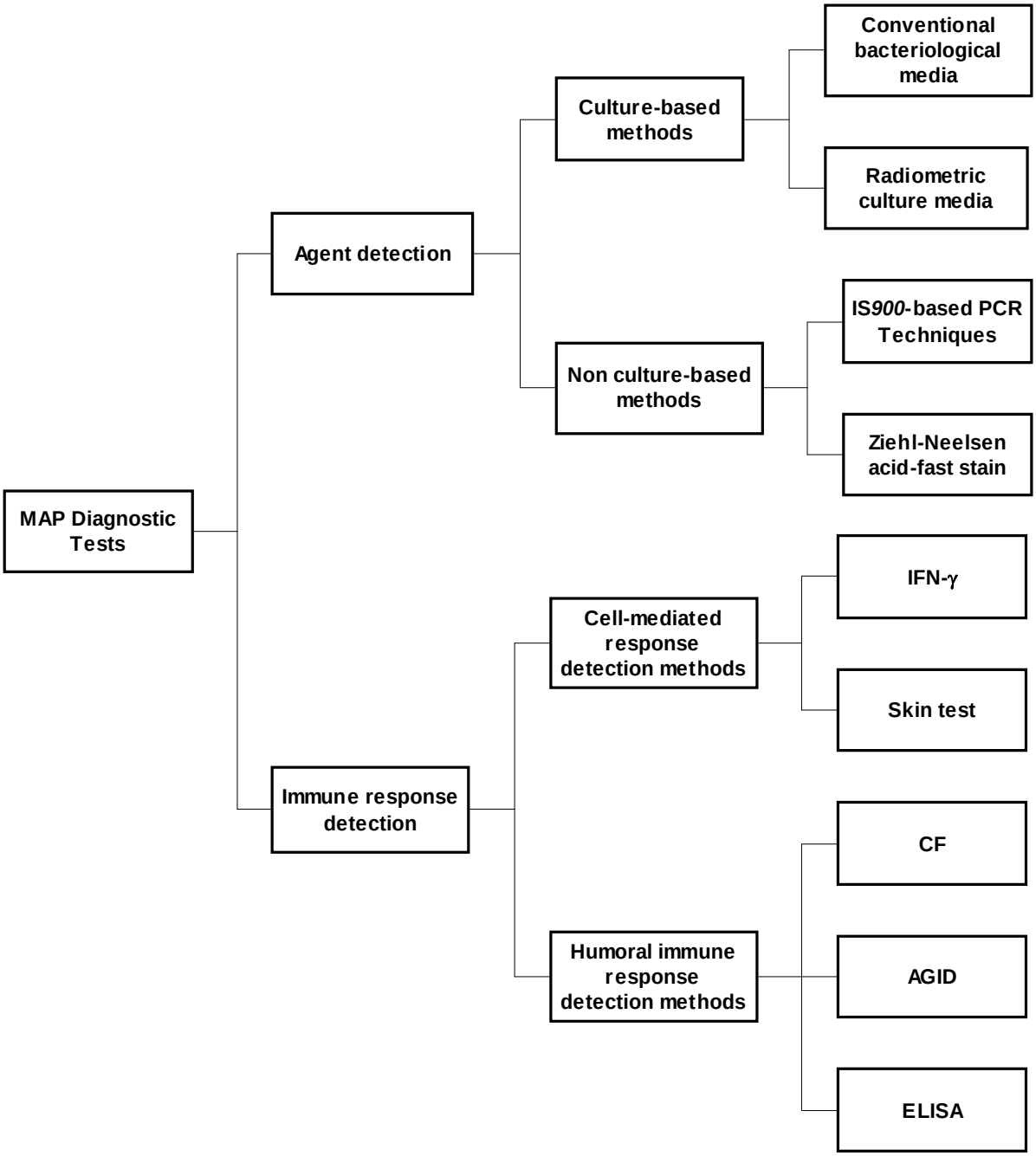

Fig. 4 – Summary of most commonly used MAP diagnostic tests. IFN-γ – Interferon Gamma; CF – Complement Fixation; AGID - Agarose Gel Immunodiffusion; ELISA - Enzyme-Linked Immunosorbent Assay (adapted from Nielsen et al.(2001).

2.7.2 Agent detection tests

Agent detection is one of the most used methods to diagnose MAP infection. Several matrices have been used to detect MAP such as faeces, milk and other tissues. Depending on the method used, the detected analyte may be the bacteria (bacterial stain – ZN, culture)

or bacteria and/or its constituents (PCR techniques). As a basic principle for agent detection, MAP must be present in the sample and available in detectable quantities. MAP isolation major problem is to ensure that MAP is present in the collected sample. About tissue culture, Whitlock et al (1996) mentioned that it may be necessary to collect samples from up to 100 sites per animal in order to obtain a positive culture of tissue specimen from an infected animal. Thus the sampling collection method, the sample processing steps and the agent detection method chosen are very important aspects to bear in mind.

2.7.2.1 Decontamination and concentration methods

In order to reduce fungal and bacteriologic contamination an appropriate decontamination procedure is necessary. MAP cells may be damaged and growth inhibited. The HPC (hexadecylpyridinium chloride) has been the most commonly used decontaminant, but others have also been reported e.g. benzalkonium chloride and oxalaic acid (reviewed in: Nielsen et al., 2001).

Pre-incubation methods and the use of antimicrobial agents have also been described. Some associations between chemical and antimicrobial agents for decontamination and culture methods are recommended (reviewed in: Nielsen et al., 2001).

To increase MAP recovery from samples concentration methods should be applied before cultivation process. Centrifugation and sedimentation methods are used but the former is described as having higher detection and lower contamination rates. Filtration is a technique used to concentrate MAP samples using the MAP cell clumping property. Immunomagnetic separation (IMS) is also used to extract MAP from heterogeneous samples. It has been used in milk samples and also prior to PCR (reviewed in: Nielsen et al., 2001).

2.7.2.2 Culture-based methods

Since its first isolation by bacteriologic culture, MAP isolation methods have evolved a lot but special growth requirements and some difficulties on the cultivation continue to be the main obstacles. In order to reduce the presence of fungi and other bacteria the decontamination step is particularly important. Isolates should be submitted to mycobactin dependency tests to differentiate it from other mycobacteria,[5] (reviewed in: Manning & Collins, 2001).

Two types of culture methods are available: conventional and radiometric. MAP growth velocity is different between methods. The conventional method takes between 12 and 16 weeks, while radiometric method takes only 5 to 8 weeks. The time difference is due to the

[5] MAP growth only on media supplemented with mycobactin J, an iron chelator required for in-vitro Map growth. The sample is streaked to media with mycobactin and to media without mycobactins, and the growth patterns are compared (reviewed in: Manning & Collins, 2001).

detection process. In the conventional method, slants of media are inspected until a colony is visible. In the radiometric method, a machine monitors sample-inoculated bottles of media for ^{14}C-labelled products of bacterial metabolism. Metabolic products are detectable before the organism forms a colony sizeable enough to be observed on standard slants of media by naked eye. Both the analytical and the diagnostic sensitivity of the radiometric method are higher than the conventional culture. The automation of the process is another advantage (reviewed in: Manning & Collins, 2001).

Herrold's Egg Yolk and Löwenstein-Jensen mediums complemented with mycobactins J are the most used mediums in conventional culture. Regarding the radiometric type, a commercial product known as the BACTEC system is used (reviewed in: Nielsen et al., 2001).

Culture methods sensitivity and specificity are dependent on several aspects. MAP tissue culture sensitivity and specificity have not been estimate thoroughly but they were reported elsewhere: sensitivity - 4% [0.2% - 15%] ; specificity – 97% [92% - 99%] (reviewed in: Anon, 2004b). Regarding faecal culture, the results were reported by animal status: Affected, Se – 70%, Sp – 100%; Infectious, Se – 74%, Sp – 100%; Infected, Se – 23% - 29%, Sp – 98% (Nielsen & Toft, 2008).

2.7.2.3 Non culture-based methods

IS*900* has been used as the MAP genetic marker on PCR techniques since the discovery of the insertion sequence. IS*900*-based PCR can be applied directly on samples or as a confirmatory step after conventional culture, radiometric culture or immunomagnetic separation processes (reviewed in: Nielsen et al., 2001).

Due to its high sensitivity and fast response it is broadly used. However it also has some limitations. False-negative reactions may occur due to the presence of certain substances such as urea, haemoglobin or heparin that may inhibit enzymatic reactions; and MAP cell wall resistance. False-positive reactions are also described and may be caused by: laboratory contamination; IS*900* sequence is not so MAP specific, as it was previously assumed – "IS*900*-like sequences" (Englund, Bolske & Johansson, 2002; Cousins et al., 1999); and amplification of DNA from dead bacteria and spheroblasts – these PCR positive results may be considered as a false-positive reaction when used together with a culture method (they will give a culture negative result) (reviewed in: Nielsen et al., 2001).

Although its drawbacks, IS*900*-based PCR is a fast technique and it is currently the method used to detect MAP infection in samples where MAP spheroblasts (viable and non-cultivable MAP forms) are present (Cousins et al., 1999; Chiodini, Vankruiningen, Thayer & Coutu, 1986).

The Se and Sp of direct PCR on faecal samples were reported: Se – from 2% [0% - 5%] to 39% [10% - 90%]; Sp – 98% [95% - 100%] (Anon, 2004b).

Another method of agent detection is by direct stain of samples followed by microscopic examination. Direct acid-fast staining of faecal samples has a low sensitivity and is very difficult to accurately distinguish MAP from non-pathogenic bacteria. Tissues direct staining is a commonly used process on samples collected at necropsy or by biopsy. Usually the Ziehl-Neelsen (ZN) acid-fast stain is the method used to highlight rod-shaped organisms (reviewed in: Manning & Collins, 2001).
MAP identification by PCR techniques and by ZN may be hampered in paucibacillary forms of disease (reviewed in: Manning & Collins, 2001).

2.7.3 Immune response detection tests

To measure an immune response, first it must have occurred at a detectable intensity regarding the test used and the timing of the testing. Measurable immunological reactions can be grouped into cell-mediated or humoral immune responses. In the former, the skin test (intradermal injection of a MAP purified protein derivate) and interferon-gamma (IFN-γ) can be used. In humoral immune responses, the agarose gel immunodiffusion (AGID) test, the complement-fixation (CF) test and the absorbed indirect enzyme-linked immunosorbent assay (ELISA) are the most commonly applied tests (reviewed in: Nielsen et al., 2001).
None of the mentioned tests is perfect (Se – 100%; Sp – 100%) and one of the major difficulties in their evaluation is the use of bacteria detection as the “gold standard” of infection. As bacteria detection methods are not perfect, the test results may be over or underestimated.
False-positive reactions in serological tests may be due to cross-reactions with other micro-organisms because the antigens used are crude and may contain antigens common to other bacteria of the order Actinomycetales. On the other hand, the animal may develop a protective immunologic response, recover after being exposed and be considered resistant. The problem of differentiating between resistant and infected animals is difficult to overcome by immunologic techniques since these methods give an indication of exposure, not infection (reviewed in: Chiodini et al., 1984).
False-negative responses may occur due to an insufficient antibody level (e.g. early infection) or to a state of anergy developed in clinical cases attributable to the protein-losing enteropathy. An animal that had responded to antigen but fail to respond in subsequent testing may be anergic to that specific antigen and infected or may have recovered from infection (Chiodini et al., 1984; reviewed in: Buergelt et al., 2004)

2.7.3.1 Humoral immune response detection methods

AGID test is considered less sensitive than ELISA and the CF. Its high specificity may be a result of the low analytical sensitivity (high-antibody detection limit). The CF test is reported to detect antibodies later than ELISA and its specificity is considered lower than AGID and ELISA (reviewed in: Nielsen et al., 2001).

ELISA is the most commonly used test to detect humoral immune response in MAP infection. Due to the pre-absorption with *Mycobacterium phlei*[6] the sensitivity is improved. Results depend on the chosen cut-off point and the diagnostic test used as standard. The key factor in the determination of the ELISA efficacy is the type of antigen used (reviewed in: Buergelt et al., 2004).
ELISA sensitivity and specificity are dependent on several factors such as stage of infection and animal status. Estimates of ELISA specificity and sensitivity for serum and milk samples were described regarding the animal status (Nielsen & Toft, 2008).

Table 1 – Range of reported sensitivity (Se) and specificity (Sp) of serum antibody ELISA (SELISA) and milk antibody ELISA (MELISA) for three animal statuses: affected, infectious and infected.

	SELISA		MELISA	
Animal Status	Se	Sp	Se	Sp
Affected	50% - 87%	No data	No data	No data
Infectious	24% - 94%	40% - 100%	21% - 61%	83% - 99%
Infected	7% - 22%	85% - 100%	39%	96% - 100%

(adapted from: Nielsen & Toft, 2008)

2.7.3.2 Cell-mediated response detection methods

Intradermal testing and detection of interferon-gamma are used to detect cell-mediated immune response in MAP infection. The former is based on the delayed-type hypersensitivity (DTH) after intradermal injection of the antigens. A MAP purified protein derivate (PPD) is inoculated and the reaction (skin thickness) is measured. This test was replaced due to the lack of specificity and the poor correlation with the infectious status of the animal (reviewed in: Nielsen et al., 2001).

[6] Saprophytic bacteria existent in intestinal surface (reviewed in: Chiodini et al., 1984).

Interferon-gamma (IFN-γ) cytokine, released by sensitized lymphocytes after exposure to appropriate antigens, is another way used to detect cell-mediated immune responses (reviewed in: Nielsen et al., 2001).

It is gaining increasing acceptance due to the possibility of detection of infected animals in an earlier stage of infection (during cell-mediated immune response stage).

Its major drawback is the time span from blood sample collection to the start of culture because it will influence the cells viability (Mikkelsen, Jungersen & Nielsen, 2009). On the other hand non-specific reactions, different responses of species to the non-specific stimulant, interpretation issues and the time consuming nature of the protocol are other weak points of this test (reviewed in: Manning & Collins, 2001).

Its characteristics were described only for the infectious animal status: Se – from 13% to 85%; Sp – 88% (Nielsen & Toft, 2008).

3 Distribution of MAP in tissues

3.1 Introduction

Among findings related to infection with MAP, lesions at the gastro-intestinal tract are the most commonly described. However, some authors have not only found gross and histopathologic lesions, but also isolated and identified MAP by culture and PCR in tissues outside the intestinal tract. Considering this aspect of MAP infection as a disseminated infection[7] a literature revision was made to: (i) identify previous studies on which isolation of MAP in tissue cultures was described; (ii) detect which tissues are more frequently analyzed and which tissues require further attention; (iii) assess the prevalence of MAP contamination in each tissue; (iv) make recommendations for a future study to assess MAP distribution in tissues in order to quantify inputs necessary to a risk assessment.

3.2 Materials and Methods

An electronically literature search was made using the Medline/Pubmed database. The search terms were paratuberculosis, Johne's or Johnes, culture, isolation, tissues, milk and beef. Due to the amount of results and the difficulty of reach the important articles through the article title or abstract, a manually literature search was made. This manual search used the study from Antognoli et al (2008) as a starting point to reach other important articles about MAP isolation by tissue culture. As inclusion criteria, MAP isolation must be done by tissue culture and the information of positives/total sampled need to be described for each tissue. Only studies regarding cattle were considered. It was decided not to stipulate more specific criteria. However, some important characteristics from each study are pointed so their objectives, study design and methodology can be understood. The main reasons for this decision were the fact that there are few published data and because the date of publication differed substantially (1929-2009) between studies, with differences on the method used to detect MAP distribution on tissues. For each study the following information was selected: clinical status, purpose of the study, husbandry type, age range, clinical status description, sample size, sampling method, cross-contamination precautions, type of infection and type of study and tissue collection.

The number of MAP positive tissues and samples collected were also recorded. For each sampling site the apparent prevalence (positives/sampled) was calculated and for each

[7] (Dennis et al., 2008; Antognoli et al., 2008; Alonso-Hearn et al., 2009; Hines et al., 1987; Pavlik et al., 2000);

prevalence estimate the 95% Wilson Score Interval (Confidence Interval) was calculated (Brown et al., 2001).

$$CI_{WSI} = \frac{p + \frac{z^2{}_{\alpha/2}}{2n} \pm z_{\alpha/2}\sqrt{[p(1-p) + z^2{}_{\alpha/2}/4n]/n}}{(1 + z^2{}_{\alpha/2}/n)}$$

Eq. 1 – Wilson Score Interval equation.

3.3 Results

A total of 48 studies met the criteria for inclusion. Study information and study results were recorded and three main tables were constructed: In Table 2 and Table 3 the study details are shown and grouped by clinical status, purpose of the study, husbandry type and age range; in Annex 1 the tissue culture results are grouped by tissue; in Table 4 and Table 5 the prevalence and 95% Confidence intervals are grouped by tissue summary – results from Annex 1. The assumptions made regarding the description of the studies are described below.

Clinical Status

The studies were firstly grouped by "clinical status" since it is relevant to know if the culture positives tissues came from an "affected animal" (with decreased production indicators and/or clinical signs), which could be rejected at the slaughterhouse based on observable signs, or if they came from an "asymptomatic/not affected" infected animal (without clinical signs and decreased production indicators). This last group of animals with possible contaminated tissues will easily enter into the food chain if no precautions are taken to detect MAP in tissues consumed by humans. In 48 studies, 1 study (Doyle, 1958) did not fit to any of these categories because the detection of MAP was only made in foetus tissues from cows clinically affected with Johne's disease; 25 studies were about "asymptomatic" animals; 9 studied asymptomatic and affected animals; 13 studies defined their studied object as "affected" animals. This last group included animals that shown clinical signs and/or changes in production indicators such as reduction of milk yield.

Some authors did not clearly specify the clinical status of the animals. Cattle were considered asymptomatic for three reasons: (1) too young assuming the usual age for manifestation of clinical signs[8]; (2) because the time between experimental exposure to the agent and slaughter was too short for the manifestation of clinical signs; (3) because the animals were "abattoir animals" approved at the *ante mortem* inspection.

[8] Although some were experimental infection studies where the exposure dose is an important factor which was not taken into account.

The reasons for categorizing the animals as asymptomatic on the studies mentioned above were: in Macdonald et al (1999) animals were culled between 21 and 29 months old; in Beard et al (2001) “no animal exhibited signs of diarrhoea or weight loss during the 6-month incubation period”; in Paine and Rankin (1961b) the oldest animal culled was 17 months old; in Larsen et al (1975) the animals were culled 150 days after exposure; in Paine and Rankin (1961a) the oldest calf was 9 months old and all cattle were culled 6 months after exposure. Animals were considered asymptomatic because they were abattoir animals in McNab et al (1991), McKenna et al (2004), Jorgensen (1965), Pavlik et al (2000), and in Whitlock et al (1997); in Ayele et al (2004) the reason to assume that animals were asymptomatic was because some were calves between 6 and 28 weeks old and others were asymptomatic breeding bulls that were culled based on a faecal culture positive test.
Most of the studies described affected animals as cattle with “clinical signs of disease” without specifying those signs. (Category CSD – “Clinical signs of Disease” - in field “Clinical Status description”).

Animals were considered in the “Both” category on the study from Huda and Jensen (2003) because they described them as “subclinically and clinically infected animals”. Some exhibited clinical signs compatible with paratuberculosis while others had signs of mastitis, lameness or reproductive disorders. The same applies to the study of Kopecky et al (1967) were some animals were culled with CSD while others were culled due to other reasons. The study by Rossiter and Henning (2001) was included in the category “Affected” because animals had a low body condition score, and were considered “at high risk of clinical infection”.

The characteristics of the reviewed studies where MAP was isolated by tissue culture are in Table 2 and Table 3 [9].

[9] C.S. – Clinical Status; Purp. – Purpose; Husb. – Husbandry; Cross-cont. – Cross-contamination; Inf. – Infection; col. – collection; Na – Not applicable; Nd – No data; Path – Pathogenesis studies; Prev – Prevalence studies; Tdist – Tissue distribution studies; Dtests – Diagnostic test evaluation studies; CSD – Clinical signs of disease; FC – Fecal culture; NR – Non-random; R – Random; E – Experimental; N – Natural; CrSe – Cross-sectional; CaSt – Case Study; CrSe F – Cross-sectional with follow-up; Rep CrSe – Repeated Cross-sectional; TC – Tissue culture; PCR – Polymerase chain reaction; < 2 years – younger than 2 years old; ≥ 2 years – with or older than 2 years old; D – Diarrhoea; W – Weight loss; Abt – Abattoir animals; M – Milk yield decreased; Rt – Routine; It – Intensive.

Table 2 – Characteristics of the reviewed studies where MAP was isolated by tissue culture (part 1).

C.S	Study Purp.	Husb. Type	Age range	Clinical Status description	Sample size	Sampling method	Cross-cont. precautions	Inf. Type	Study Type	Tissue col. Type	Reference
Affected	Path	Dairy	< 2 y	D, W	21	Na	Nd	E	E	Rt	(Thorel, Pardon, Irgens, Marly & Lechopier, 1984)
	Prev	Both	≥ 2 y	Abt, W	539	NR	Nd	N	CrSe	It	(Rossiter & Henning, 2001)
		Dairy	≥ 2 y	D, W	11	NR	Yes	N	CrSe	It	(Giese & Ahrens, 2000)
	Tdist	Both	≥ 2 y	CSD	6	NR	Yes	N	CaSt	It	(Larsen & Kopecky, 1970)
		Dairy	≥ 2 y	D, W, FC+	1	Na	Yes	N	CaSt	It	(Larsen et al., 1981)
				D, W, ELISA+	40	NR	Yes	N	CrSe	It	(Antognoli et al., 2008)
				CSD	34	NR	Yes	N	CrSe	It	(Doyle, 1954)
				D, W, M	1	Na	Nd	N	CaSt	It	(Hines et al., 1987)
				D, W	7	NR	Yes	N	CaSt	It	(Koenig et al., 1993)
				CSD	26	NR	Yes	N	CrSe F	It	(Taylor et al., 1981)
				D, W	4	NR	Nd	N	CaSt	It	(Alexejeff-Goloff, 1929)
				CSD	24	NR	Yes	N	CaSt	It	(Lawrence, 1956)
				CSD	4	NR	Yes	N	CaSt	It	(Rohde & Shulaw, 1990)
Asymptomatic	Dtests	Dairy	≥ 2 y	As	29	Na	Nd	Both	E	Rt	(McDonald, Ridge, Hope & Condron, 1999)
			Nd	As	1493	NR	Yes	N	CrSe	It	(Jayarao et al., 2004)
				As	24	R	Yes	N	CrSe	It	(Paolicchi et al., 2003)
	Path	Beef	< 2 y	As	12	Na	Nd	E	E	It	(Stabel, Palmer & Whitlock, 2003)
		Dairy	< 2 y	As	18	Na	Nd	E	E	Rt	(Beard et al., 2001)
				As	4	Na	Nd	E	E	Rt	(Saxegaard, 1990)
				As	21	Na	Nd	E	E	It	(Payne & Rankin, 1961b)
				As	26	Na	Yes	E	E	It	(Gilmour, Nisbet & Brotherston, 1965)
			≥ 2 y	As	5	Na	Nd	E	E	It	(Rankin, 1961a)
			All	As	8	Na	Nd	E	E	Rt	(Larsen et al., 1975)
				As	16	Na	Nd	E	E	It	(Payne & Rankin, 1961a)
	Prev	Both	≥ 2 y	Abt	400	R	Nd	N	CrSe	Rt	(Smith, 1954)

Table 3 – Characteristics of the reviewed studies where MAP was isolated by tissue culture (part 2).

C.S	Study Purp.	Husb. Type	Age range	Clinical Status description	Sample size	Sampling method	Cross-cont. precautions	Inf. Type	Study Type	Tissue col. Type	Reference
Asymptomatic	Prev	Both	≥ 2 y	Abt	400	NR	Nd	N	CrSe	Rt	(McNab, Meek, Duncan, Martin & Vandreumel, 1991)
				As	7540	R	Nd	N	Rep CrSe	Rt	(Merkal, Whipple, Sacks & Snyder, 1987)
		Dairy	≥ 2 y	Abt	100	R	Nd	N	CrSe	It	(Chiodini & Vankruiningen, 1986)
				Abt	984	R	Nd	N	CrSe	Rt	(McKenna et al., 2004)
			All	Abt	1110	NR	Nd	N	CrSe	Rt	(Jorgensen, 1965)
			Nd	As	86	NR	Yes	N	CrSe	It	(Ayele et al., 2005)
				As	211	NR	Nd	N	CrSe	It	(Pillai & Jayarao, 2002)
	Tdist	Both	≥ 2 y	Abt	611	NR	Nd	N	CrSe	It	(Pavlik et al., 2000)
		Dairy	≥ 2 y	As, Abt, FC+	171	NR	Yes	N	CrSe	It	(Whitlock et al., 1996)
				Abt, FC+	86	NR	Yes	N	CrSe	It	(Sweeney et al., 1992b)
				As	58	NR	Yes	N	CaSt	It	(Sweeney et al., 1992a)
			All	As	14	NR	Yes	N	CrSe	It	(Ayele, Bartos, Svastova & Pavlik, 2004)
			Nd	As	126	NR	Yes	N	Rep CrSe	It	(Streeter et al., 1995)
Both	Dtests	Dairy	≥ 2 y	D, W, M, other	15	NR	Yes	N	CrSe	It	(Huda & Jensen, 2003)
	Path	Dairy	≥ 2 y	As, CSD	23	Na	Nd	Both	E	It	(Taylor, 1953)
				As, M, CSD	9	Na	Nd	E	E	It	(Rankin, 1961b)
			All	As, D, M	6	Na	Nd	E	E	It	(Rankin, 1958)
	Prev	Dairy	≥ 2 y, Nd	CSD	33	NR	Yes	N	CrSe	It	(Smith, 1960)
	Tdist	Dairy	≥ 2 y	Abt, CSD	407	NR	Yes	N	CrSe	It	(Seitz et al., 1989)
				CSD, other	148	NR	Nd	N	CrSe	Rt	(Kopecky, Larsen & Merkal, 1967)
		Both	≥ 2 y	M, ELISA+, D, W, Faecal PCR+	47	NR	Yes	N	CrSe	It	(Alonso-Hearn et al., 2009)
		Nd	Nd	Abt, D, W	21	NR	Yes	N	CrSe	It	(Brady et al., 2008)
Na	Tdist	Na	Na	Na	24	NR	Nd	N	CrSe	It	(Doyle, 1958)

Purpose of the study

The studies analyzed were categorized by their main objective and divided into 4 groups: Diagnostic test evaluations, Pathogenesis, Prevalence and Tissue distribution studies.
The studies included in the first group performed tissue culture as the confirmatory method to other diagnostic tests.
The Pathogenesis studies were all experimental studies with experimental infection. Their main objective was to evaluate the characteristics of the agent and different aspects of the disease pathogenesis.
When their main goal was to estimate the prevalence in a specific geographical region by detecting MAP in tissues, e.g. prevalence studies at slaughterhouse, they were included in the Prevalence group.
If the isolation of MAP from a specific location or the demonstration of a generalized infection were the aim of the study, they were considered as an evaluation of MAP distribution in tissues.
This division was made because different study purposes and different methods used to assess the MAP distribution lead to different study results.

Husbandry type

Studies were also grouped in dairy or beef cattle since different market purposes determine different management schemes and consequently different environmental factors that may predispose to infection. In the studies of Larsen and Kopecky (1970), Smith (1954) and McNab et al (1991) the husbandry type was assumed to be dairy and beef because these studies were made at a slaughterhouse. So there are some odds that both types of cattle were slaughtered.
Merkal et al (1987), Pavlik et al (2000), Rossiter and Henning (2001) differentiate their results by type of husbandry. Alexejeff-Goloff (1929) and Lawrence (1956) did not specify the type of study animals. However at least one was dairy so all were considered as dairy cattle. Most studies were on dairy cattle: 38 of 48 (79%).

Age range

As age affects disease progression it may also influence the dissemination of MAP in tissues. The age at slaughter was used to difference two age ranks: < 2 years old and ≥ 2 years old. When no clear information about the age of the studied animals was given, animals were considered older than 2 years old if the study was done at an abattoir. In the studies from Alexejeff-Goloff (1929), Alonso-Hearn et al (2009), Doyle (1954), Giese and

Ahrens (2000), and Taylor et al (1981) cattle were also considered older than 2 years old since the authors reported all were clinically affected animals. In 29 of 48 (60%) of studies animals were ≥ 2 years old.

Clinical status description

If this information was available an attempt was made to describe the clinical status of the animals. When clinical signs different than the classical signs of paratuberculosis were stated they were classified as "other". In Huda and Jensen (2003) "other" signs were mainly reproductive disorders, mastitis and lameness. In Kopecky et al (1967) they were mastitis, respiratory disorder and lumpy jaw. When there was mention of clinical signs without a description of them, the studies were classified as CSD.

Sample size

In Table 2 and Table 3 sample size represents the maximum number of animals with tissues analyzed. In Annex 1 the number of samples collected by each tissue is given[10]. In some studies, tissue collection was not carried out in all sampled animals either by author's option or because of contaminated/invalid samples.

Sampling method

The sampling method was categorized in random or non-random. The sampling method used by Merkal et al (1987) and Smith (1954) was considered random because both were abattoir studies where the authors avoid the selection of animals from the same herd by systematic sampling (the first collect samples with 1 hour interval and the second on different days). Most of the studies were considered "non-random" because the selection was based on previous animal/herd positive diagnostic tests, observation of clinical signs of disease or convenience sampling. Only 4 in 48 (10%) were considered random sampling studies.

Cross-contamination precautions

They were considered when the author demonstrated awareness of the possibility of cross-contamination for example, faecal contamination in milk during milk collection; bacterial contamination between tissues and materials; faecal contamination in tissues collection at

[10] For each animal several tissues could be gathered. For each tissue only one sample per tissue was considered. In cases where several samples were gathered from the same tissue and gave different results - some positive and others negative, it was recorded only as one positive sample (or if all negative – one negative sample).

the abattoir; and aseptic procedures in sample collection[11]. It was present in 23 of 48 (48%) studies.

Type of tissue collection

Tissue collection was described as intensive or routine. If only gastro-intestinal samples were collected, studies were marked as “Routine”, if gastro-intestinal and/or other tissues were collected, the studies were designated as “Intensive”.

3.3.1 Animal population characterization

From all 15004 studied animals, 22485 samples were analyzed giving an average of 1.5 samples per animal. 2297 samples were MAP culture positive (10.3%).
Approximately 90% of the studied animals were asymptomatic animals (77% of the collected samples), while only 5% were affected animals (13% of the collected samples) (“Both” and “NA” categories together accounted for 5% of animals and 10% of samples).
The majority of the animals – 76% (64% of samples) was tested in “prevalence studies”, 13% of the total animals (18% of samples) were tested in “tissue distribution studies”, 1% (9% of samples) in “pathogenesis studies”, and 10% (9% of samples) in “diagnostic test studies”.
Concerning the husbandry type, approximately 60% were dairy cattle (67% of samples) and 26% beef cattle (23% of samples) (“Both”, “ND” and “NA” represented together 14% of animals and 10% of samples).
In relation to age approximately 83% were ≥ 2 years old (81% of samples) and only 4% were < 2 years old (8% of samples) (“NA” and “ND” summed 13% of animals and 11% of samples).
Regarding the type of infection, 98% of animals (90% of samples) had natural infection while only 1% (8% of samples) was submitted to experimental infection. Only 1% of animals (2% of samples) belong to studies where both types of infection were present. Regarding the study type, if we regroup the several cross-sectional studies in a single “general” cross-sectional study category they ensemble 98% of the analyzed animals (89% samples), while experimental studies represented 1% (10% samples) and case studies also 1% (1% samples).
Although the number of studies with random sampling was low, they covered 60% of the tested animals (47% of samples). Non-random sampling was used for 39% of animals and 43% of samples; “NA” was 1% of animals and 10% of samples.

[11] e.g. Non-gastrointestinal tissues collected first; udder cleaning and disinfection procedures in milk collection; sterile materials.

Due to the lack of information about cross-contamination precautions and aseptic procedures, the studies that provided this information covered only 18% of the tested animals (23% of samples).

Though most studies were categorized as based upon an intensive method of tissue collection, they represented 29% (46% samples) while routine methods represented 71% of the tested animals (54% samples).

3.3.2 Characterization of analyzed tissues

A total of 56 different tissues were cultured for MAP and were categorized into 10 different tissue sections: 9 sections of gastro-intestinal mucosa (16%), 8 sections of gastro-intestinal lymphoid tissue (14%), 16 types of other lymphoid tissue (29%), 4 internal organs (7%), genitourinary tract (14%), and 11 other tissues (20%). The number of positive and samples were summarized per tissue and the prevalence were calculated (Annex 1). Whenever possible the tissues were regrouped in larger tissues sections in Table 4 and Table 5.

Table 4 – Results from Annex 1 summarized by tissue[12] (Part 1). Prevalence (Prev.) and 95% Confidence interval (CI) of MAP culture isolation by tissue and larger tissue sections.

Intestinal Mucosa Summaries	**Positives**	**Samples collected**	**Prev.**	**95% CI**	
Colon muc.	32	96	33.3%	24.7%	43.2%
Cecum muc.	40	121	33.1%	25.3%	41.8%
Ileo-cecal valve muc.	119	397	30.0%	25.7%	34.7%
Jejunum muc.	54	183	29.5%	23.4%	36.5%
Rectum muc.	18	100	18.0%	11.7%	26.7%
Duodenum muc.	20	115	17.4%	11.5%	25.3%
Pylorus muc.	6	37	16.2%	7.7%	31.1%
Ileum muc.	160	1272	12.6%	10.9%	14.5%
Abomasum muc.	4	44	9.1%	3.6%	21.2%
Others	131	188	69.7%	62.8%	75.8%
Intestinal Mucosa	584	2553	22.9%	21.3%	24.5%

GI Lymphoid Tissue Summaries	**Positives**	**Samples collected**	**Prev.**	**95% CI**	
Colic LN	8	12	66.7%	39.1%	86.2%
Jejunal LN	13	30	43.3%	27.4%	60.8%
Ileal LN	14	42	33.3%	21.0%	48.4%
Mesenteric LN	181	617	29.3%	25.9%	33.0%
Jejunal Peyer's Patches	5	18	27.8%	12.5%	50.9%
Duodenal LN	4	29	13.8%	5.5%	30.6%
Cecal LN	8	100	8.0%	4.1%	15.0%
Ileocecal LN	436	10757	4.1%	3.7%	4.4%
Others	23	47	48.9%	35.3%	62.8%
GI Lymphoid tissue	692	11652	5.9%	5.5%	6.4%
Others	253	1035	24.4%	21.9%	27.2%
GI tract	1529	15240	10.0%	9.6%	10.5%

Head Lymphoid Tissue Summaries	**Positives**	**Samples collected**	**Prev.**	**95% CI**	
Suprapharyngeal LN	19	37	51.4%	35.9%	66.6%
Submaxilar LN	18	41	43.9%	29.9%	59.0%
Retropharyngeal LN	34	122	27.9%	20.7%	36.4%
Mandibular LN	6	51	11.8%	5.5%	23.4%
Tonsil	14	178	7.9%	4.7%	12.8%
Parotid LN	0	37	0.0%	0.0%	9.4%
Others	27	40	67.5%	52.0%	79.9%
Head Lymphoid tissue	118	506	23.3%	19.8%	27.2%

[12] Only one sample per tissue per animal was used. In each tissue summary, the number of "Positives" and "Samples collected" per tissue also represent the number of sampled and positive result animals. (e.g. In colon mucosa 96 samples from 96 animals were collected and 32 gave positive result which means 32 positive animals). One exception is muscle tissue, where 167 samples do not correspond to 167 but to 87 animals, because the 3 different muscles from Antognoli et al (2008) were here grouped (corresponding to 40 animals and not to 120 animals). On the other hand, e.g. 2553 samples collected from tissues from intestinal mucosa (summary) do not represent 2553 analyzed animals because in the same study, the same animal may have had various tissues from intestinal mucosa analyzed.

Table 5 – Results from Annex 1 summarized by tissue (Part 2). Prevalence (Prev.) and 95% Confidence interval (CI) of MAP culture isolation by tissue and larger tissue sections.

Other LN Summaries	**Positives**	**Samples collected**	**Prev.**	**95% CI**	
Hepatic LN	94	221	42.5%	36.2%	49.1%
Pulmonary LN	32	82	39.0%	29.2%	49.8%
Mediastinal LN	28	134	20.9%	14.9%	28.5%
Supramammary LN	52	251	20.7%	16.2%	26.2%
Precrural LN	6	37	16.2%	7.7%	31.1%
Iliac LN	11	69	15.9%	9.1%	26.3%
Prescapular LN	18	127	14.2%	9.2%	21.3%
Popliteal LN	19	660	2.9%	1.9%	4.5%
Superficial Cervical LN	7	539	1.3%	0.6%	2.7%
Bronchial LN	0	37	0.0%	0.0%	9.4%
Others	13	19	68.4%	46.0%	84.6%
Other LN	398	2682	14.8%	13.5%	16.2%
All Lymphoid tissue	1090	14334	7.6%	7.2%	8.0%

Internal Organs Summaries	**Positives**	**Samples collected**	**Prev.**	**95% CI**	
Lung	20	119	16.8%	11.2%	24.5%
Spleen	32	201	15.9%	11.5%	21.6%
Kidney	13	98	13.3%	7.9%	21.4%
Liver	54	798	6.8%	5.2%	8.7%
Genitourinary tract Summaries	**Positives**	**Samples collected**	**Prev.**	**95% CI**	
Prostate	4	7	57.1%	25.0%	84.2%
Seminal vesicles	9	20	45.0%	25.8%	65.8%
Ovaries	5	18	27.8%	12.5%	50.9%
Uterus	24	163	14.7%	10.1%	21.0%
Epididymis	2	14	14.3%	4.0%	39.9%
Testes	3	22	13.6%	4.7%	33.3%
Bulbourethral gl.	2	17	11.8%	3.3%	34.3%
Bladder	1	12	8.3%	1.5%	35.4%
Other Tissues Summaries	**Positives**	**Samples collected**	**Prev.**	**95% CI**	
Semen	3	3	100.0%	43.8%	100.0%
Uterine flush fluid	3	4	75.0%	30.1%	95.4%
Others	30	55	54.5%	41.5%	67.0%
Collection chamber flush fluid	2	6	33.3%	9.7%	70.0%
Mammary Gland	11	56	19.6%	11.3%	31.8%
Plasma	1	7	14.3%	2.6%	51.3%
Pharynx	4	37	10.8%	4.3%	24.7%
Colostrum	10	126	7.9%	4.4%	14.0%
Fetus	30	515	5.8%	4.1%	8.2%
Muscle	8	167	4.8%	2.4%	9.2%
Milk	99	2091	4.7%	3.9%	5.7%
Mammary Gland flush fluid	0	7	0.0%	0.0%	35.4%
All tissues analyzed	2297	22485	10.2%	9.8%	10.6%

3.3.2.1 MAP prevalence in tissues

For all tissues and animals tested the overall prevalence of MAP tissue contamination was 10.2% [9.8% - 10.6%].

Regarding lymphoid tissue the overall prevalence was 7.6% [7.2% - 8.0%], while gastro-intestinal lymphoid tissue was 5.9% [5.5% - 6.4%] and other lymphoid tissue 14.8% [13.5% - 16.2%]. 74% of lymphoid tissue samples belong to Ileocecal LN.

The gastro-intestinal tract had an overall prevalence of 10.0% [9.6% - 10.5%] while intestinal mucosa had 22.9% [21.3% - 25.5%]. In whole GI tract, 77% of samples belong to GI Lymphoid tissue.

Semen was the “tissue/body fluid” with the highest prevalence 100% [43.8% - 100%] followed by the uterine flush fluid 75% [30.1% - 95.4%]. However, this was a consequence of the small number of samples tested as it is demonstrated by the amplitude of the confidence interval. If sample size consists of a minimum of 100 animals sampled, then the tissues with highest prevalence rates were the hepatic LN (42.5% [36.2% - 49.1%]), the cecum mucosa (33.1% [25.3% - 41.8%]), the ileocecal valve mucosa (30% [25.7% - 34.7%]) and the jejunal mucosa (29.5% [23.4% - 36.5%]).

The tissues that had most samples analyzed were ileocecal LN (10757 samples; prevalence 4.1% [3.7% - 4.4%]), milk (2091 samples; prevalence 4.7% [3.9% - 5.7%]), ileum mucosa (1272 samples; prevalence 12.6% [10.9% - 14.5%]) and liver (798 samples; prevalence 6.8% [5.2% - 8.7%]).

The tissues with most positive samples were the ileocecal LN (436 positive samples; prevalence 4.1% [3.7% - 4.4%]), mesenteric LN (181 positive samples; prevalence 29.3% [25.9% - 33.0%]) and ileum mucosa (160 positive samples; prevalence 12.6% [10.9% - 14.5%]). The last four mentioned tissues were also the most analyzed (between 12 and 16 studies).

If we focus on animal body regions, 46% of the studies analyzed the gastro-intestinal tract (including gastro-intestinal lymph nodes), 54% non gastro-intestinal tissues and 42% the lymphoid tissue (including gastro-intestinal lymph nodes).

Only two studies (Alonso-Hearn et al., 2009; Antognoli et al., 2008) analyzed muscle tissue. In the first only diaphragm muscle was tested, in the second three different muscles were analyzed but MAP was only isolated on heart. MAP contamination prevalence was 4.8% [2.4% - 9.2 %].

Milk contamination was found in 4.7% [3.9% - 5.7%] of samples studied (10 studies).

The information relative to age and husbandry type was also collected in order to try to find an association between these factors and the presence of MAP in specific tissues. The statistical analyses were made but it was not possible to take any conclusion due to the different study designs which result in inconsistent results.

3.4 Discussion

Since the first description of paratuberculosis, *Mycobacterium avium* subspecies *paratuberculosis* organisms have been isolated from several tissues, faeces and body fluids. Initially, only suspected animals with clinical symptoms were tested but with the knowledge of the subclinical stage of infection, attempts have been made to look for MAP organisms in tissues of animals not showing disease characteristic clinical signs.
MAP isolation has been used to prove MAP localization in a specific tissue, to confirm the result of other diagnostic tests for paratuberculosis or even to estimate the disease prevalence in a population. Recently, MAP isolation has been used to evaluate its occurrence in tissues bearing in mind a possible link between paratuberculosis and Crohn's disease.

In this work some studies with results about MAP isolation in tissues were used. Comparison of the results need to be made with caution, because they came from studies published in the last eight decades, in different populations, using different methods of investigation the same problem and with different strains of MAP.
Experimental infection studies were allowed in the inclusion criteria because they analyzed a variety of tissues that otherwise would be without any information. Moreover their influence on the global results is not relevant (if they were excluded it will not change significantly the result). Their inclusion can be criticized because they do not represent the reality and the infectious dose that animals were exposed may differ substantially of the dose in natural infection.

Though PCR is a technique easier to perform and faster than culture, the studies that isolated MAP by PCR were not included because PCR detects residual DNA and dead organisms, not making any differentiation between viable and non viable organisms, and thus has a low specificity for detection of tissues not containing live MAP.
On the other hand, there is the problem of the possible existence of "paucibacillary forms" of infection, where the low number of the microorganisms may limit PCR techniques (Amemori et al., 2004). However, it can be a powerful tool if the PCR positive or PCR inconclusive tissues would then be submitted to a tissue culture for confirmation.

In order to properly evaluate the MAP distribution in tissues, further studies are needed. Some aspects of the reviewed studies will be criticized and suggestions will be given that may guide further studies.
To proceed with an epidemiological study the main objective must be specified. Regarding the possibility of MAP occurrence as a public health threat two types of studies should be

considered: pathogenesis study and prevalence study. In the former, an experimental study to assess MAP tissues susceptibility could be made. Guidelines for MAP experimental challenge models are described elsewhere (Hines II et al., 2007). On the other hand, an investigation of a well characterized population of naturally infected animals (with or without clinical signs, with positive or negative diagnostic tests), based upon a rigorous sanitary inspection and the collection of tissue samples could be a very fruitful option.

With a better understanding of the host susceptibility, distribution and degree of contamination of MAP in tissues, further searches for MAP in naturally infected animals could be more precise and cost-effective. From a risk manager point of view this information should be assessed by specific risk groups with the objective to implement specific measures to reduce the risk of a contaminated cattle product entering the food chain.

Further studies suggestions

Hereafter, the discussion will focus on relevant aspects of a study to assess MAP distribution in tissues in a risk assessment view.

Population level - animal selection

Some authors suggested that the distribution of MAP in tissues is dependent on the time of uptake (Momotani et al., 1988; Sigurdardottir, Press & Evensen, 2001) and on the host immune response (Stabel, 2000a; Bendixen, 1978). If we consider that MAP dissemination in animal tissues is dependent on the prevailing type of immune response and that these responses correspond to different stages of infection, then the distribution of MAP in tissues may be associated with the infection stages. In the estimation of regional susceptibility of MAP in tissues, the stage of infection should be taken into account because sampling on different stages may produce different results. With these stage-specific results the best sampling sites could be investigated and recommended also by infection stages. Therefore, on a study to assess MAP distribution, the study population should be representative of the target population and it should include cattle in different stages of infection. To do a stratified sampling the animal population must be very well characterized and the distribution of animals by age, infected, infectious and affected animals must be available. For each of these statuses, the relation between the results of the diagnostic tests and the MAP distribution in tissues should be investigated.

Some studies analyzed in this work tried to assess the relation between positive culture results and clinical status, husbandry type and age range. In early studies that confirmed the existence of MAP outside the gastro-intestinal tract, cattle were selected based on the existence of clinical signs or the shedding of the agent in faeces. This made the

disseminated infection appear to be a characteristic of a late-stage of infection (Whitlock & Buergelt, 1996; Huda & Jensen, 2003; Buergelt et al., 1978; Pavlik et al., 2000). However, recent studies report that this type of infection could also be found in asymptomatic animals (Antognoli et al., 2008; Brady et al., 2008).

Approximately 10.8% [9.71% - 11.92%] of the samples collected from affected animals were positive while asymptomatic animals had approximately 7.5% [7.14% - 7.93%] of their samples positive ($P < 0.001$). It was expected to observe a larger difference between groups. However, the proportion of samples collected by each group have to be considered (asymptomatic – 76.4%, affected – 13.5% of the collected samples). It was not possible to assess the proportion of infected animals by clinical status neither the proportion of infected animals with positive cultures, because the majority of animals were selected based on previous infection demonstration.

Two studies evaluated the effect of the husbandry type. Merkal et al (1987) observed a significant difference of prevalence between husbandry types of 2.9% for dairy cattle and 0.8% for beef cattle (difference 2.1% ± 0.35%). Pavlik et al (2000) estimated prevalence of dairy cattle were 44.9%, in beef cattle 31.1% and in dual-purpose cattle 29.0%. Both research teams attributed these differences to the differences in the management system. In our study it was not possible to assess the association between husbandry type and positive culture results for each tissue due to different study designs. However, the proportion of positive samples on dairy herds 13% and on beef herds was only 2% ($P < 0.001$). These values are in agreement with the findings of Merkal et al (1987) and Pavlik et al (2000). These differences are likely to reflect environmental disease determinants that increase the odds of infection, rather than an intrinsic host predisposition to contain MAP in a certain tissue. If this relation should be evaluated, the sampled animals should represent the distribution of each husbandry type on the concerned population.

Some studies evaluated the factor age as a resistance or a susceptibility to infection. However, the studies that evaluated MAP distribution in tissues did not explore the age influence on the probability of MAP occurring in certain tissues. Age may be a factor of major importance and further studies are needed not only to improve the understanding of the relation between age and susceptibility to infection but also the relation between specific age groups and MAP presence in specific tissues.

Animal level – tissue selection

Despite the variety of the objectives in the reviewed studies, all isolated MAP on a specific tissue. As expected by the date range of the publications, the methodology of cultivation, isolation and identification of the organism varied between studies.
The objective of each study determined which tissues were collected. While some authors limited the tissue analysis to the gastro-intestinal tract (routine method), others collected also extra-gastrointestinal tissues (intensive method).
The choice of the tissues to be collected should be based on the distribution of MAP infection, which is not completely understood. In fact some aspects need further clarification, for example: (i) which tissues outside the gastro-intestinal tract have more probability of being infected, (ii) the time of infection when these tissues become infected and (iii) how the organism disseminates inside the host.

Regarding the nature of clinical signs and the present knowledge of the disease, the gastro-intestinal tract was the region most studied for MAP isolation in tissues: 31 in 48 studies, 12753 in 15004 animals, and 19968 in 22485 samples collected. The majority of paratuberculosis lesions have been described in distal ileum and at the ileocecal valve (Buergelt et al., 1978), jejunum and ileum (Brady et al., 2008), and from the duodenum to the rectum (Taylor, 1953).
Concerning MAP isolation, in the study from Pavlik et al (2000), where distribution of MAP in the tissues collected was compared, the probability of detection of MAP infection in gastro-intestinal related tissues was 90%. In Amemori et al (2004)[13] distribution of MAP in intestinal mucosa and its associated LN was studied thoroughly. They calculated a positive culture proportion and suggested that the best gastro-intestinal related places to detect MAP were between jejunum mucosa to ileocecal-valve, and the distal jejunal LN, the proximal ileum LN and the cecum LN. Observing our results, 70% of the positives cultures from gastro-intestinal tract were included in the regions between jejunum and cecum (though, these regions correspond also to 91% of all analyzed). In addition, Peyer's patches had been described as the "entry portal" for the agent (Momotani et al., 1988) and are distributed over the jejunum and the ileum (Landsverk et al., 1991). Regarding these arguments, in the case of a selective sampling of gastro-intestinal tissues, the mucosa and the associated lymph nodes from jejunum to cecum should be considered the key sampling sites.

[13] Study not included in the analysis because the authors haven't expressed the results as required in the inclusion criteria. 63 animals older than 2 years were analyzed, selected at slaughter, positive by fecal culture between 1995 and 2000. 890 samples were collected from mucosa and LN between duodenum and rectum. Suspicious samples were confirmed by PCR IS*900*.

Although there is no detailed description of MAP dissemination mechanism in tissues outside gastro-intestinal tract, the existence of a sporadic bacteraemia has been suggested. Some authors demonstrated MAP in the mononuclear cell-rich fraction of blood and in several tissue fluids (Koenig et al., 1993; Barrington et al., 2003; Buergelt & Williams, 2004; van der Giessen et al., 1995)[14]. It has also been shown that infected macrophages may not have effective intracellular killing mechanisms and that acid-fast bacilli may be carried to distant sites by blood-borne or lymph-borne mononuclear phagocytes (Bendixen, Bloch & Jorgensen, 1981). The access of MAP to the circulation can be via draining lymphatics, contiguous lymph nodes, thoracic duct or by direct invasion of blood vessels (Bendixen et al., 1981).

Several authors isolated MAP from non-gastrointestinal tissues (Annex 1), but only few studies researched the relation between disseminated and gastro-intestinal infection. Pavlik et al (2000) observed that when the infection was detected in parenchymatous organs (10% probability of detection) it was also detected in gastro-intestinal tract. Antognoli et al (2008) found disseminated infection in 21/28 (75%) and gastro-intestinal infection in 7/28 (25%) of the infected animals (28/40). Within the animals with disseminated infection, the liver (10/21) and the hepatic LN (17/21) were the tissues where MAP was most isolated.
According to our analysis (Table 4 and Table 5), the tissues outside the gastro-intestinal tract where MAP was most isolated were: hepatic LN, mediastinal LN, supramammary LN, lung, spleen, uterus and prescapular LN. Milk should also deserve special attention because it is one of the animal products most consumed by humans and the assessed prevalence was 4.7% [3.9% - 5.7%].

There is little knowledge about MAP excretion in milk of infected cows (Sweeney et al., 1992a) such as shedding quantities, timing of the beginning of milk shedding or the dynamics of MAP shedding in milk (continuous or intermittent). If an intermittent shedding exists it may be an obstacle to MAP detection by faecal culture like it happens on faeces. Another point to bear in mind is the time between the beginning of shedding in milk and the time of MAP detection. If milk is commercialized before milk culture result is known, MAP may enter the food chain. If the relation between faecal excretion, antibody production and MAP shedding in milk is understood, precautionary measures could be implemented regarding the milk collection of infectious animals.
Another way to address this problem was adopted by the Dutch Milk Quality Assurance Programme. A programme to analyze and to reduce bulk milk tank MAP concentration

[14] The studies from van der Giessen et al (1995), Barrington et al (2003) were not included in the analysis because MAP demonstration was done by PCR.

instead of an individual animal approach is on use. They considered that the major cause for MAP occurrence in milk is faecal contamination rather than individual excretion, due to the huge quantities of viable MAP on faeces (Weber, Nielen, Velthuis & van Roermund, 2008).

Regarding muscle, Antognoli et al (2008) and Alonso-Hearn et al (2009) were the only ones to search for the presence of MAP in this tissue. Muscle tissue is very important because together with milk it is the main bovine product consumed by humans. To our knowledge, the mechanism of dissemination of MAP from gastro-intestinal sites to muscle, the timing of muscle infection, MAP quantities and specific muscle susceptibility are unknown. When assessing the prevalence of MAP organisms in muscle, the results should be stratified by infection stage and obtained from a very well characterized population. It would also be interesting to evaluate the relation between MAP existence in muscles and the animal clinical condition (assessing the most important clinical signs, the capacity of detecting them and culling the animal), husbandry type, age range and the presence of gross lesions. With this information, the risk could not only be assessed regarding the status of the animal confirmed by laboratory tests, but also by observable factors in the slaughterhouse.

Relation between macroscopic lesions and MAP isolation

An association between existence of Paratuberculosis macroscopic lesions and the isolation of MAP organisms was established by several authors (Brady et al., 2008; Amemori et al., 2004; Dennis et al., 2008). Though tissues should be investigated for the presence of macroscopic lesions, as their existence depends on several factors and they are more likely to occur on advanced stages of the disease, they are not sufficiently specific to confirm paratuberculosis infection (González et al., 2005; Amemori et al., 2004; Dennis et al., 2008; Buergelt et al., 1978). The existence of focal, multifocal and diffuse forms of lesions together with the possibility of MAP existence on tissues without lesions should be considered when deciding the number of samples collected per tissue. However, the relation between observable MAP gross lesions in gastro-intestinal tract and MAP dissemination to other tissues has not yet been studied. If there is a relation this information could be used at slaughterhouse environment. The non clinical animals accepted in *ante mortem* inspection but showing gross lesions could be detected and rejected in *post mortem* inspection. Inspector capacity of detecting infected cattle, non-clinical but with gross lesions animals needs also to be assessed.

Cross-contamination

Regarding the possibility of cross-contamination between tissues, tissues and materials, and faecal contamination, some precautions must be taken during sample collection. An aseptic technique should be preferred (tissues that have more probability of being contaminated should be collected last; materials used should be sterilized between tissues). If the collection is made at a slaughterhouse several aspects should be taken into account: the knowledge of cattle anatomy and aseptic techniques for sample collection, of the person in charge of collection (a veterinary should be preferred), and the abattoir line time (it should not compromise the normal operations of the abattoir).

Sample processing

The resistance of MAP to adverse conditions is known, however, no published studies demonstrate the effect of tissue freezing on MAP recovering by tissue culture (Hines II et al., 2007). This aspect should be investigated. Important aspects such as decontamination, isolation, identification, confirmation and quantification methods have been described by some authors (Hines II et al., 2007; Whittington, 2009)
In our analysis, MAP was isolated by the recent and oldest laboratory techniques. This implies different sensitivities and specificities, one of our major drawbacks.

Results

The results should be presented by number of positives and/or quantification/concentration of organisms, and number of tissues sampled. If several samples are made on the same tissue in the same animal and give different results, the final result of the tissue (positive or negative) should be reported. All results should be specified by groups analyzed (by age, by stage of infection, etc).

3.5 Conclusions

Though the relation between Crohn's Disease in humans and MAP in cattle remains unclear (Waddell et al., 2008), based on the precautionary principle, it is important to improve the understanding of the MAP occurrence on tissues that may enter into the food chain. Although several authors isolated MAP by culture on a diversity of tissues, to our knowledge, a study with the aim of identifying the probability of MAP occurrence and quantification on cattle tissues, on a representative sample, has not been done.

During the analysis of the different methodologies used by different studies, problems with sampling and MAP recovery were found. Due to the chronic characteristics of the disease and the lack of sensitivity and specificity of the diagnostic tests, the characterization of an animal population regarding each animal stage of infection is a very hard task. However, this information is needed to make some recommendations from a risk management point of view, both to reduce MAP dissemination on tissues and to reduce the presence of MAP contaminated tissues in food products. Regarding MAP isolation methods, the tissue culture was preferred rather than PCR techniques since the former is the only method that allows for the identification of viable and cultivable forms of the organism (which could be an important factor if a relation between diseases is confirmed). However, tissue culture also has some drawbacks: it is a slow process; it may give false positives results; and MAP quantification is hampered by MAP propensity to clump. Hence, when the most probable tissues contaminated with MAP will be known, PCR techniques will be used, as fast and highly sensitive methods to exclude MAP-infected tissues from the food chain.

The results of our work could not be used to point the likelihood of each tissue to contain MAP due to limitations discussed previously. However, our results could be use to set priorities on the choice of tissues to be sampled on future studies to assess MAP distribution on tissues. The statistics of tissues more consumed by humans is another way to establish priorities for tissue's study. On the other hand, we found that few studies investigated the existence of MAP on muscle and milk by tissue culture. Regarding frequency of consumption of these tissues they should be targeted on future investigations.

After the characterization of the occurrence and the quantification of MAP on tissues, a further step should be the development of *ante mortem* diagnostic tests to identify live animals with MAP on tissues that will be consumed by humans. With this knowledge, cattle could be defined as infectious not only regarding their potential for disease transmission to other animals (like they are by MAP in faeces or colostrum), but also because of their potential hazard to humans. At slaughterhouses could also be interesting to have a test for

MAP detection on specific tissues in order to approve or reject carcasses. Another approach would be to reject certain tissues from high risk animals.

Briefly, further studies are needed to characterize by infection stage the occurrence of MAP on animal tissues, bearing in mind the possible link between Crohn's Disease and MAP, as a potential food safety hazard to consumers.

3.5.1 Study results

Hereafter our study results will be briefly summarized.

3.5.1.1 Previous studies on MAP tissue culture isolation

The studies where MAP was isolated by tissue culture that fit the inclusion criteria are assembled on Table 2 and Table 3.

- The tissues where MAP was most investigated were:
  - Ileocecal LN – 16 studies;
  - Mesenteric LN and Ileum mucosa – 14 studies;
  - Liver – 12 studies;
  - Mediastinal LN, Hepatic LN, Spleen and Milk – 10 studies.
- Tissue which further investigation is required:
  - Muscle – 2 studies;
  - Milk – 10 studies;
  - Other tissues commonly consumed by humans.

3.5.1.2 MAP prevalence in tissues

The prevalence of MAP tissue contamination estimated in our study is summarized on Table 4 and Table 5.

- On our literature review MAP was found in:
  - Intestinal mucosa;
  - Gastro-intestinal lymph nodes;
  - Head lymph nodes;
  - Thoracic and abdominal lymph nodes;
  - Lung, spleen, kidney and liver;
  - Genitor-urinary tract tissues;
  - Other "tissues/body fluids" such as semen, plasma, colostrum, milk, mammary gland flush fluid, collection chamber flush fluid, uterine flush fluid, mammary gland, foetus, pharynx and muscle.

- Tissues where MAP was most frequently isolated[15]:
  - Intestinal mucosa: cecum, ileocecal valve and jejunum;
  - Gastro-intestinal lymphoid tissue: mesenteric LN, cecal LN and ileocecal LN;
  - Head lymphoid tissue: retropharyngeal LN and tonsil;
  - Other LN: hepatic LN, mediastinal LN and supramammary LN;
  - Internal organs: lung, spleen and liver;
  - Genitourinary tract: uterus;
  - Other tissues: colostrum, foetus, muscle and milk.
- Tissues frequently consumed by humans where MAP was most isolated:
  - Milk – 4.7% [3.9% – 5.7%];
  - Muscle – 4.8% [2.4% - 9.2%].

3.5.2 Study limitations

Our study and results have several limitations and drawbacks that are briefly described below:

- Manual and electronic literature search instead of a systematic literature review approach;
- MAP isolation only by tissue culture instead of PCR;
- MAP tissue prevalence was assessed irrespective of cattle infection status;
- MAP prevalence may not be representative and it should not be compared between tissues due to different study designs and methodologies.

3.5.3 Knowledge gaps of MAP distribution in tissues

After the literature revision several knowledge gaps and lacks of information were found:

- Detailed mechanism of MAP distribution and tissues colonization;
- Relation between paratuberculosis symptoms and MAP disseminated infection;
- Relation between diagnostic test results and MAP distribution in tissues;
- Relation between observable MAP gross lesions and the degree of MAP dissemination;
- MAP prevalence on specific tissues by stage of infection.

[15] Considering the cut-off minimum of 100 samples by tissue ordered by decreasing prevalence.

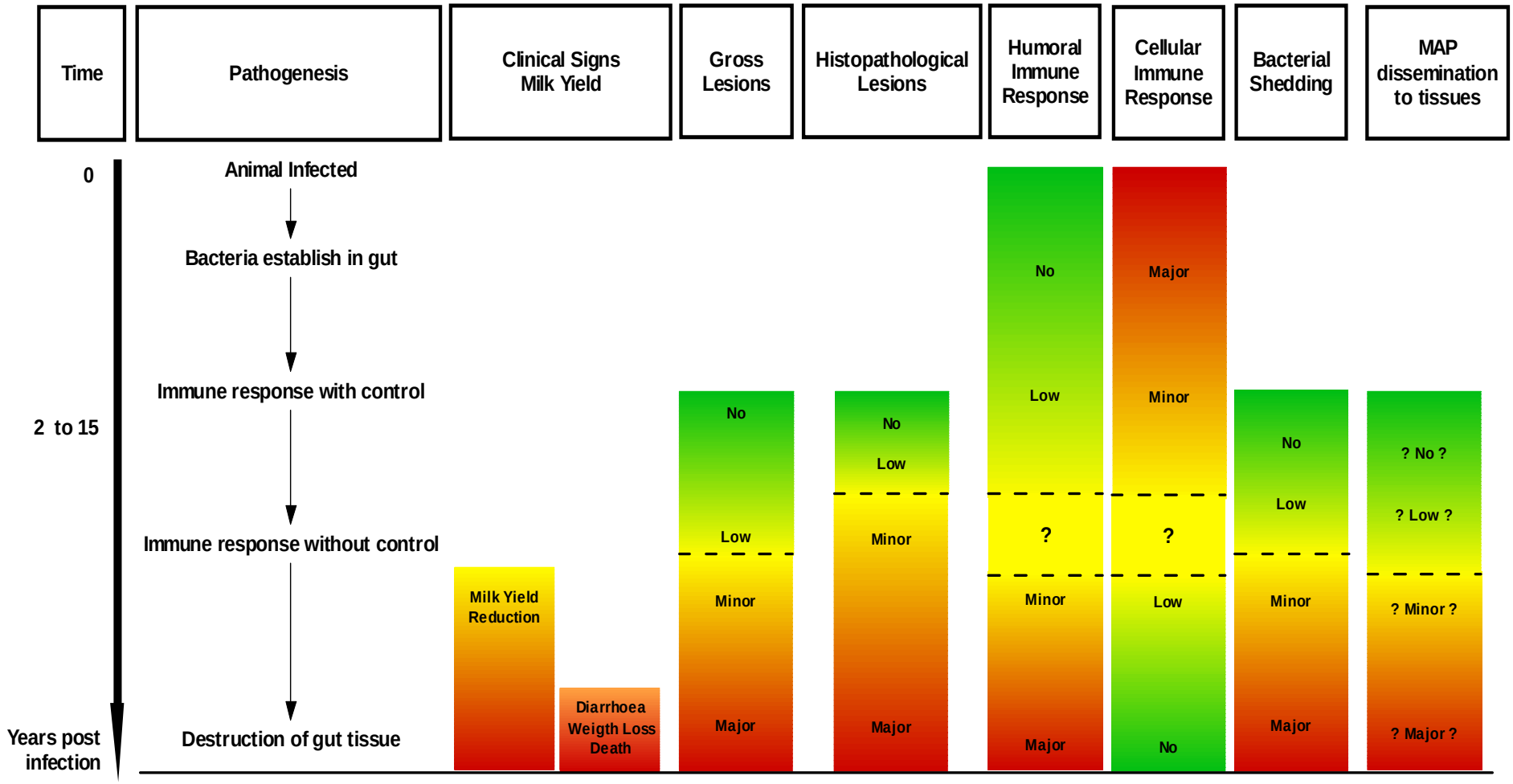

Fig. 5 – Schematic representation of pathogenesis of MAP infection and Clinical Signs, Gross Lesions, Histopathological Lesions, Humoral and Cellular Immune Responses, Bacterial Shedding and MAP dissemination to tissues. The point in time when the event pass from “Low” to “Minor” is not well known. Regarding the immune responses, the point in time when a cellular immune response shift to a humoral immune response is unknown. The relation between MAP dissemination to tissues and the previous columns need further investigation.

3.5.4 Future research

Regarding our study limitations and the current scientific knowledge gaps founded some further approaches are proposed:

- A MAP distribution in tissues assessment study:
  - Study population:
    - Should be representative of the target population;
    - Should include cattle on different stages of infection.

  - Key sampling sites to detect MAP infection[16]:
    - Gastro-intestinal tissues: mucosa and associated lymph nodes from region between jejunum and cecum;
    - Non gastro-intestinal tissues[17]: hepatic LN, retropharyngeal LN, mediastinal LN, supramammary LN, lung, spleen, uterus.

  - Cross-contamination precautions:
    - Aseptic technique:
      - Highly contaminated tissues should be collected last;
      - Sterilization of materials between collecting procedures;
      - Staff training on sample collecting procedures, animal anatomy and aseptic techniques.

- Further studies needed:
  - To make available the mentioned knowledge gaps;
  - Develop and improve diagnostic tests for direct MAP detection on specific tissues.

[16] To assess MAP distribution in tissues, key sampling sites are dependent on study objectives.

[17] Considering the cut-off minimum of 100 samples by tissue ordered by decreasing prevalence.

4 Risk assessment conceptual model

Risk analysis is a process constituted by 4 components: hazard identification, risk assessment, risk management and risk communication (Anon, 2004a; Anon, 2007).
Risk assessment is used to estimate, to evaluate, to discuss and to document the risk of an adverse event as well as its mitigation (Anon, 2004a). As referred above, in order to better understand the possibility of MAP sources to humans, a release assessment should be done. The release assessment, in The World Organisation for Animal Health (OIE) terminology, describes the biological pathways necessary to understand the problem, to identify the parameters involved and to define how parameters are interrelated.

4.1 Hazard Identification

Regarding the purpose of this risk assessment the hazard considered is the *Mycobacterium avium* subspecies *paratuberculosis* (MAP) despite the absence of an irrefutable causal relationship between MAP and Crohn's Disease. The characteristics and details about MAP and MAP infection were already described before in this dissertation.

4.2 Release Assessment

At the starting point of a risk assessment, the risk question is formulated. In our specific case, the risk question is: "**What is the probability of occurrence of MAP on unprocessed beef and milk from specific cattle risk groups?**". To address this question a risk model pathway should be designed and the likelihood and the quantity of MAP should be estimated afterwards. Two risk model pathways will be described: one for milk and other for muscle tissue. For each one, the assumptions, the required data and the data knowledge gaps will be pointed out.

4.3 Risk model pathway – Milk

The isolation of MAP from aseptically collected milk samples means that infected milking cows can shed MAP in their milk (Alexejeff-Goloff, 1929; Streeter et al., 1995; Sweeney et al., 1992a; Taylor et al., 1981). Despite some authors studied the mechanism of MAP infection in the gastro-intestinal tract, the way MAP spread within the host and its relation with different stages of infection remains unclear. Thus, the mechanism of MAP shedding is not fully understood (Sweeney et al., 1992a). However it may be dependent on animal characteristics and its stage of infection. Regarding this assumption, the information required

to assess the likelihood and the quantity of MAP presence on unprocessed milk is under mentioned. To use stochastic models in the risk assessment these data should be available by distributions instead of point estimates.

In order to assess the likelihood and the quantity of MAP on milk, the processes that allow MAP from an infected dairy cow to occur in the individual milk should be evaluated. As this is a risk model pathway for a release assessment the end-point considered will be the cow's individual milk. In the design of this pathway some assumptions were made:

- The existence of a ParaTB control programme. The characteristics of the Danish control programme were used;
- Only dairy herds provide milk for human consumption;
- Only animals ≥ 2 years old provide milk for human consumption (assumption made based on the normal low proportion of animals < 2 years that provide milk and their unlikely shedding of MAP organisms in milk);
- MAP shedding in milk is dependent on animal characteristics and stage of infection;
- Regarding the herds not in the ParaTB Programme it is considered that the animals aren't systematically tested, therefore, there is a lack of information regarding the herd and animal status. The between-herd and within-herd true prevalences and the probability of MAP dissemination to milk could be theoretical and literature-based values;
- Herds included in the ParaTB Programme have measures implemented to reduce paratuberculosis prevalence, therefore the probability of be an infected herd and have infected animals is different from the herds not in the ParaTB Programme;
- Within a herd in the ParaTB Programme there is always the possibility of existence of some non tested animals. They will be assumed as having the same distribution of risk groups as the tested animals considering that the reasons for non-testing would possibly be caused by in-herd practical questions and that they will not affect the intrinsic probability of MAP excretion in milk;
- For a herd be considered Not Infected, a representative sample of herd's animals must have been tested and give test negative results. Animals that had negative test results are considered non infected and consequently no MAP dissemination to milk occurs;
- It is assumed that the test is made on individual milk instead on the bulk milk tank, the test positive results for MAP are considered a possible hazard to humans and the milk should be discarded not representing any risk.

The Fig. 6, Fig. 7 and Fig. 8 are schematic representations of the milk risk model pathways.

Fig. 6 - Milk risk model pathway part 1 – herd characteristics.

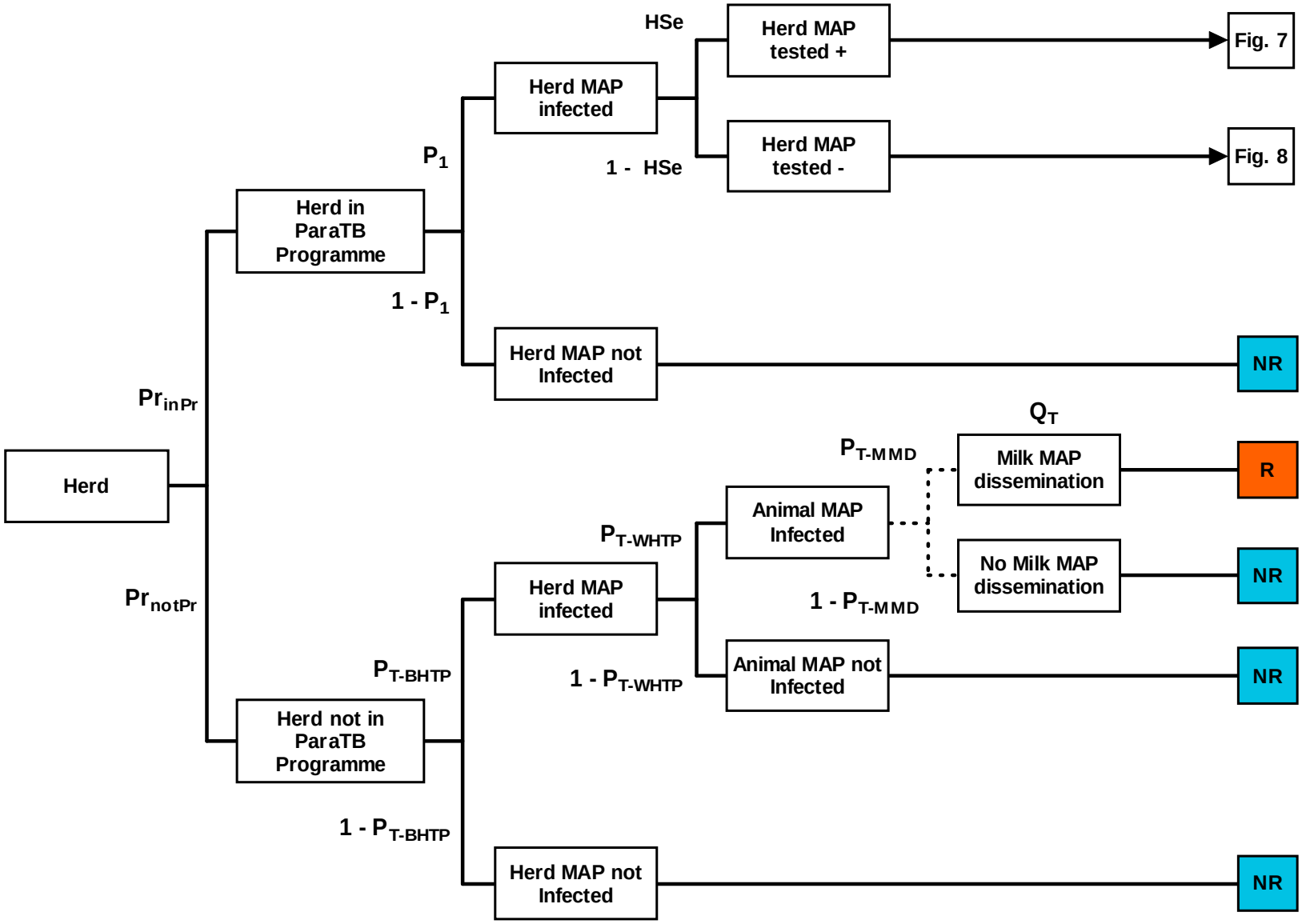

Fig. 7 – Milk risk model pathway part 2 – animal characteristics given that an infected herd gives test positive result. The dotted line represents the lack of data to support the relation between MAP dissemination to milk and the previous boxes in the figure.

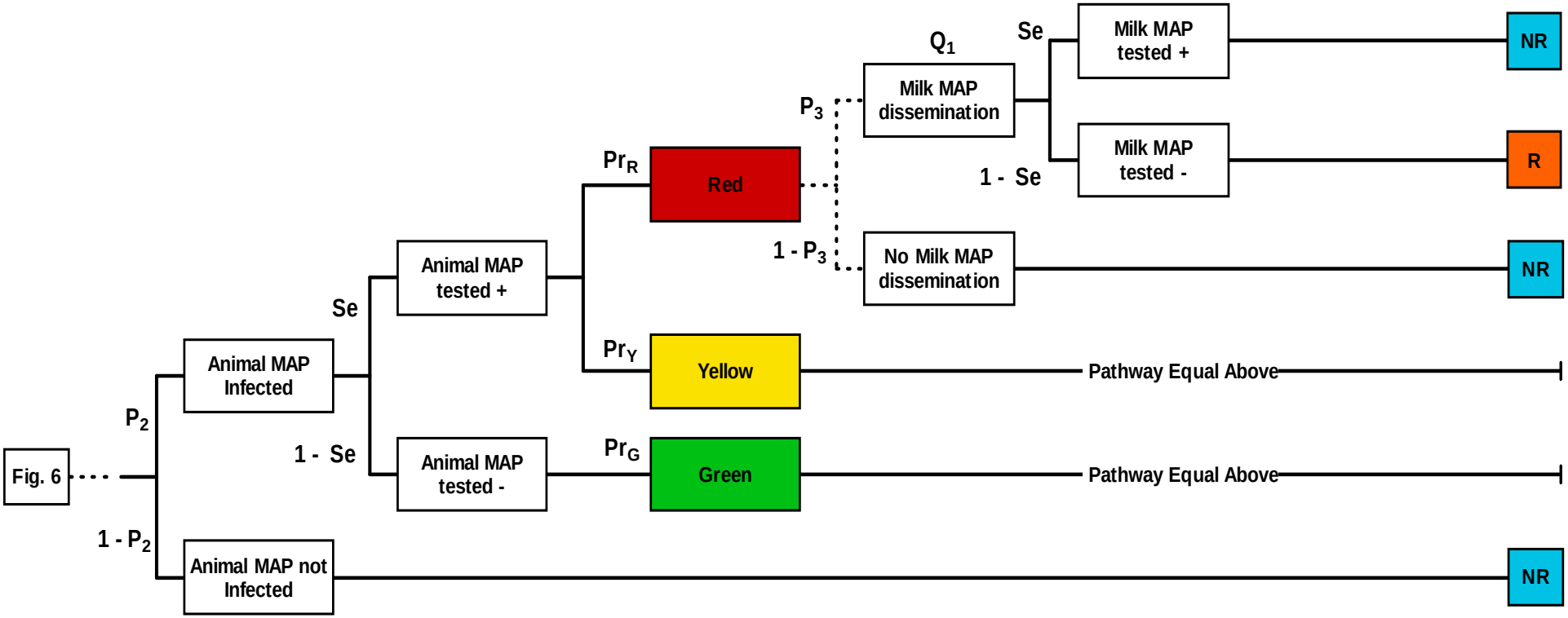

Fig. 8 – Milk risk model pathway part 2 – animal characteristics given that an infected herd gives test negative result. The dotted line represents the lack of data to support the relation between MAP dissemination to milk and the previous boxes in the figure.

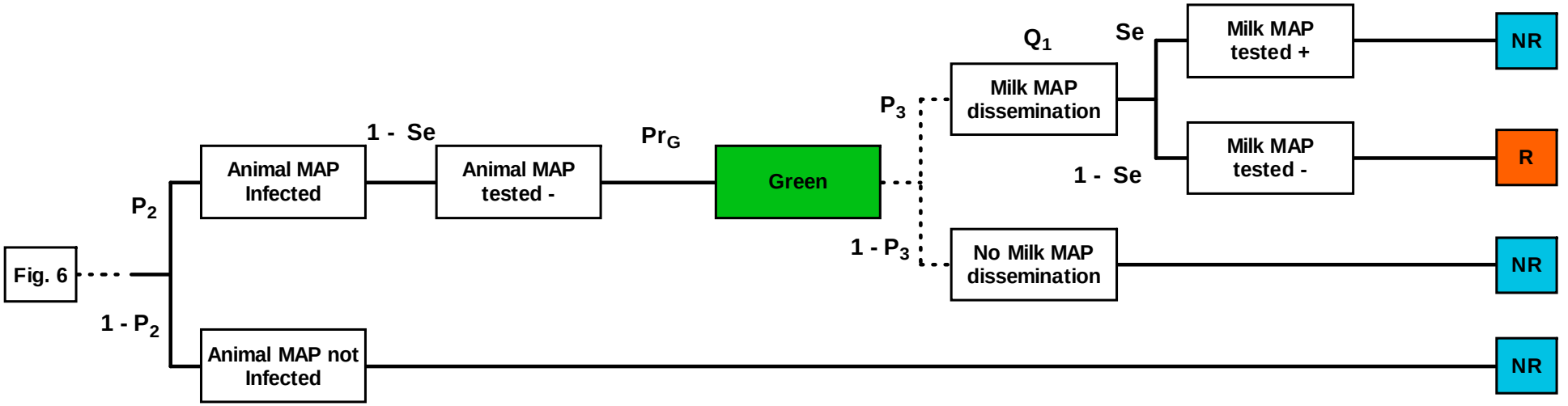

Table 6 - Milk risk model pathway legend

Box name	Description
Herds in ParaTB Programme	Due to thoroughly animal testing in a ParaTB Programme, information about Herd Infection and Test Status should be available for herds in the ParaTB Programme. The ParaTB prevalence will also be influence by the existence of the control programme.
Herds not in ParaTB Programme	
Herd MAP infected	Herd Infection Status. In a Non Infected Herd is considered that all animals are Not Infected and consequently there is No Risk of MAP occurring in Milk.
Herd MAP not infected	
Herd MAP tested +	Herd Test Status. An Infected Herd can be Test Positive or Negative regarding the test sensitivity.
Herd MAP tested -	
Animal MAP Infected	Animal Infected Status. In a Non Infected Animal there is No Risk of MAP occurring in Milk.
Animal MAP not Infected	
Animal MAP tested +	Animal Test Status. An Infected Animal can be Test Positive or Negative regarding the test sensitivity. In a Non Infected Animal there is No Risk regardless the Animal Test result.
Animal MAP tested -	
Red	Animal test results characterization representing the different animal infection stages. Colour scheme used in the Danish ParaTB Programme regardless the lack of knowledge regarding the relation between antibody profile and MAP dissemination to Milk.
Yellow	
Green	
MAP disseminated to Milk	MAP occurrence in Milk by MAP dissemination to milk in an infected animal. This MAP occurrence should be related with the animal infection stage and the programme colour scheme. If MAP has not disseminated to milk there is No risk regardless the Milk test result.
MAP not disseminated to Milk	
Milk MAP tested +	Milk Test Status for the MAP occurrence. Milk where MAP dissemination has occurred can be MAP Test Positive or Negative regarding the test sensitivity. A milk MAP tested positive may be considered as No Risk if this milk is discarded not entering the food chain.
Milk MAP tested -	
Pathway Equal Above	Above pathway repeats.
NR	No Risk
R	Risk

Table 7 - Milk risk model pathway description: list of variables and data availability.

Variable	Variable description	Comments	Availability
P_1	Probability of a herd be MAP Infected	Between-herd true prevalence for the herds in the ParaTB Programme. The measures implemented due to the existence of a ParaTB Programme will influence this prevalence.	Data available for Denmark (but only apparent prevalence)
$1 – P_1$	Probability of a herd be MAP not Infected		
P_2	Probability of an animal be MAP Infected	Within-herd true prevalence.	Data available
$1 – P_2$	Probability of an animal be MAP not Infected		
P_3	Probability of MAP dissemination to milk	Prevalence of MAP occurrence in milk by MAP dissemination to milk. This information should be related with animal infection stage. To our knowledge this information is not currently available.	**Not available**
$1 – P_3$	Probability of MAP non dissemination to milk		
Q_1	Quantity of MAP occurrence in milk by MAP dissemination to milk	Quantity of MAP occurrence in milk by MAP dissemination to milk. This information should be related with animal infection stage. To our knowledge this information is not currently available.	**Not available**
$P_{T\text{-}BHTP}$	Probability of a herd be MAP Infected	Theoretical between-herd true prevalence for the herds not in the ParaTB Programme (could be literature-based).	Data available
$1- P_{T\text{-}BHTP}$	Probability of a herd be MAP not Infected		
$P_{T\text{-}WHTP}$	Probability of an animal be MAP Infected	Theoretical within-herd true prevalence for the herds not in the ParaTB Programme (could be literature-based).	Data available
$1- P_{T\text{-}WHTP}$	Probability of an animal be MAP not Infected		
$P_{T\text{-}MMD}$	Probability of MAP dissemination to milk	Theoretical prevalence of MAP occurrence in milk by MAP dissemination to milk (could be literature-based).	**Not available**
$1- P_{T\text{-}MMD}$	Probability of MAP non dissemination to milk		
Q_T	Quantity of MAP occurrence in milk by MAP dissemination to milk	Theoretical quantity of MAP occurrence in milk by MAP dissemination to milk (could be literature-based).	**Not available**
HSe	Herd sensitivity of the test used	An Infected Herd can be considered Test positive or negative regarding the test sensitivity.	Data available
1 – HSe			
Se	Sensitivity of the test used	An Animal or Milk can be considered Test positive or negative regarding the test sensitivity.	Data available
1 – Se			
Pr_{inPr}	Proportion of herds in the ParaTB Programme	It is important to make this differentiation because it will influence the ParaTB prevalence and the available information about infection and test status of the “herd”, “animal” and “milk”.	Data available
Pr_{notPr}	Proportion of herds not in the ParaTB Programme		
Pr_G	Proportion of cows in the Green risk group	It is important to make this differentiation because different proportions of risk groups will influence the final MAP occurrence in Milk. Colour scheme used in the Danish ParaTB Programme regardless the lack of knowledge regarding the relation between antibody profile and MAP dissemination to Milk.	Data available
Pr_Y	Proportion of cows in the Yellow risk group		
Pr_R	Proportion of cows in the Red risk group		

4.3.1 Data availability and risk description

Data availability

Most information about the herd and the animal characteristics should be available on the databases of the ParaTB control programme. However, in the case of the Danish ParaTB control programme not all information is available with the detail proposed by this risk model pathway:

- Only herds in the ParaTB programme have information about the herd and animal infection and test status. This information for herds not in the ParaTB programme is not available.
- The allocation of animals into different risk groups is based upon animal's milk antibody profile. To our knowledge, the mechanism of MAP dissemination to udder is not understood so the relation between the degree and quantity of MAP dissemination to milk and the antibody response is unknown. Then there is no data available for the different probabilities and quantity of MAP excretion on milk by different risk groups.

Risk description

In the context of this risk model pathway it is assumed that there is no risk to MAP entering the food chain when herds, animals and milk are considered not infected and when milk is considered infected, test positive and discarded not entering the food chain.

There is risk of MAP entering the food chain in the specific case of infected herds not in the ParaTB Programme (because there are infected animals that could be excreting MAP into milk and this information is not available) and in milk where MAP excretion had occurred and gives test negative result.

In this risk model pathway all information is about cow's individual milk. If the bulk tank milk should be considered it will be important to differentiate a scenario where milk is grouped by risk groups or a scenario of no separation between risk groups with all milk being mixed in the bulk tank. Regarding the actual Danish ParaTB Programme, because there aren't measures implemented considering MAP as a hazard to humans, all milk is entering the food chain despite the cow's risk group. In the scenario where there is no separation of the milk produced by different risk groups, their individual probabilities should be added and the proportion of cows on each risk group will affect the final probability and quantity of MAP occurrence in the bulk tank milk. Regarding MAP quantity and considering all milk mixed in the bulk tank, the dilution effect will also need to be incorporated in the model.

The MAP quantities in faeces and the probability of faecal contamination during milk collection are other important aspects, related with milking hygiene routines and milk collection, but are out of the scope of this work. However, in order to evaluate the MAP added to milk by faecal contamination two values must be known: the quantity of faeces that may contaminate the milk during milk collection and the quantity of MAP in this amount of faeces regarding the distribution of risk groups of animals on the herd.

Depending on the objectives of the risk assessment and of the scope of the risk question, units to each parameter should be assigned. For example, the evaluation of each probability and MAP quantity could be made by litre of milk.

As an example, the final probability of MAP occurrence on milk MAP test negative, from infected cows, in the Red risk group, from herds MAP test positive would be the product of: the proportion of herds in the ParaTB programme; the probability of the herd be MAP infected; the herd sensitivity of the test; the probability of the animal be MAP infected; the sensitivity of the test used; the proportion of cows in the Red risk group; the probability of MAP dissemination to milk in cows of the Red risk group and the (1 – sensitivity) of the test used. In this scenario the final risk would also consider the quantity of MAP excreted by this Red risk group cows.

4.4 Risk model pathway – Muscle

Regarding MAP presence on muscle tissue, in the literature search described previously, only two articles were found (Alonso-Hearn et al., 2009; Antognoli et al., 2008). In the first study diaphragm muscles were investigated (6 positive animals in 47 animals). In Antognoli et al. (2008) only three muscles were investigated: heart muscle (2 positives in 40 animals), *Longissimus colli* (0/40) and *Extensor carpi radialis* (0/40). As mentioned for the milk model pathway, MAP spreading throughout the host and its relation with different stages of infection is unknown. The same applies to the probability of MAP occurrence and the quantity of MAP on muscle.

To assess the likelihood and the quantity of MAP in muscle, the factors that influence MAP presence in muscle of slaughtered cattle should be analyzed. Thus the end-point of the release assessment will be the muscle tissue at the end of the slaughter line – unprocessed beef. In the construction of this pathway some assumptions were made:

- The existence of a ParaTB control programme. In this case the characteristics of Danish control programme will be used, however, the Danish Paratuberculosis Programme was designed for Dairy cattle only;
- The characteristics of Danish milk testing scheme and risk group's colours will be used to differentiate risk groups in cattle ≥ 2 years old from Dairy herds. Another hypothetical differentiation in risk groups (High/Medium/Low) will be used for non dairy cattle and dairy cattle < 2 years old (not providing milk). These risk groups could be assessed using other diagnostic test more suitable to these specific animals;
- Dairy and non dairy herds provide beef for human consumption;
- MAP presence in muscle is dependent on animal characteristics and their stage of infection;
- Regarding the herds not in the ParaTB Programme it is considered that the animals aren't systematically tested, therefore, there is lack of information regarding the herd and animal status. The between-herd and within-herd true prevalences and the probability of MAP dissemination to milk could be theoretical literature-based values;
- Herds included in the ParaTB Programme have measures implemented to reduce paratuberculosis prevalence, therefore the probability of be an infected herd and have infected animals is different from the herds not in the ParaTB Programme;
- Within a herd in the ParaTB Programme there is always the possibility of existence of some non tested animals. They will be assumed as having the same distribution of risk groups as the tested animals considering that the reasons for non-testing would possibly be caused by in-herd practical questions and that they will not affect the intrinsic probability of MAP dissemination to muscle;

- For a herd be considered not infected, a representative sample of herd's animals must have been tested and give test negative results. Animals that had negative test results are considered non infected and consequently no MAP dissemination to muscle occurs;
- It is assumed that an animal in the Red risk group or in the High risk group showing clinical signs at the *ante mortem* inspection will be rejected not representing any risk.
- It is assumed that muscle tested positive results for MAP are considered a possible hazard to humans and the muscle should be rejected not representing any risk.

In the following figures is illustrated a schematic representation of the muscle risk model pathway.

Fig. 9 - Muscle risk model pathway part 1 - herd characteristics.

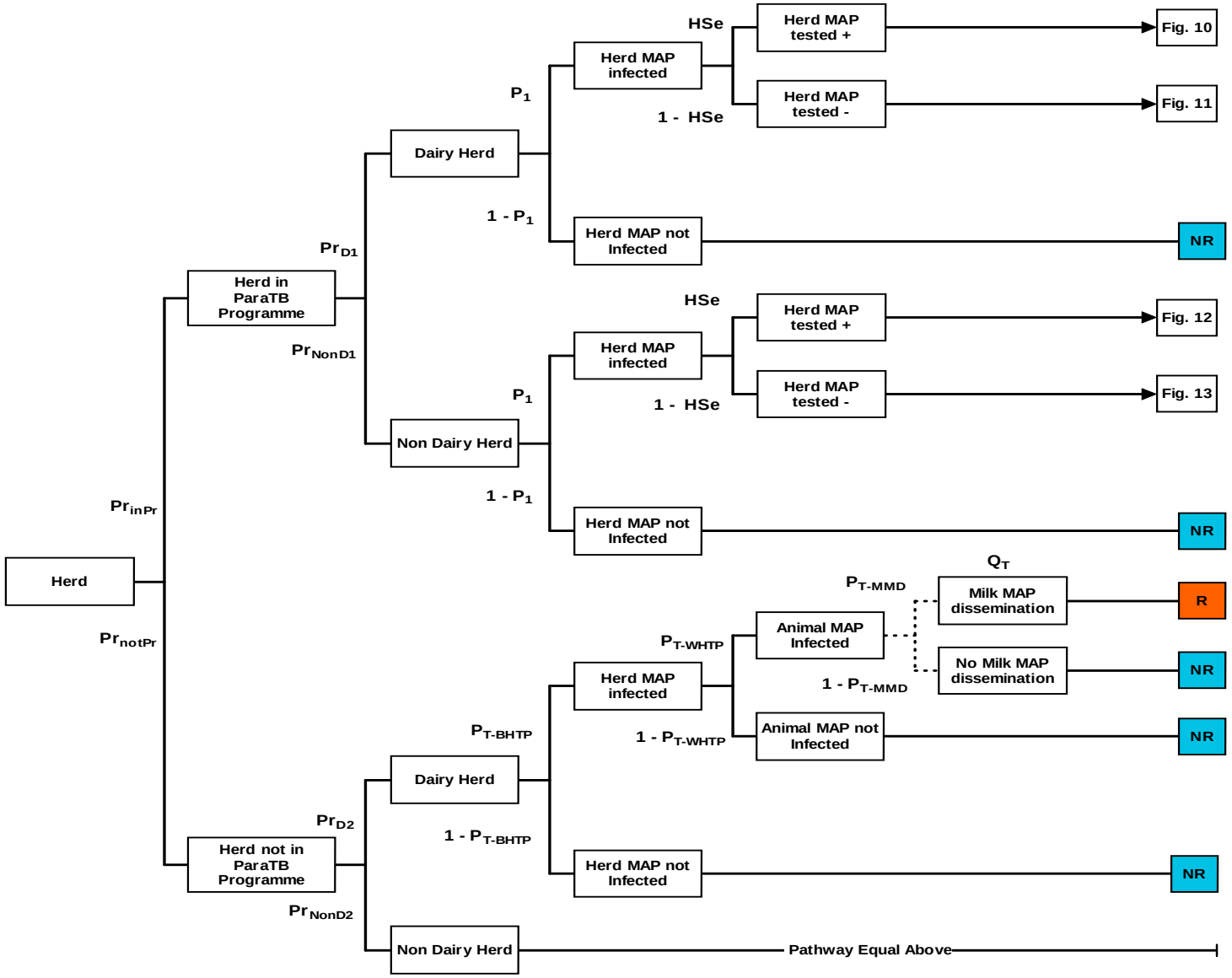

Fig. 10 – Muscle risk model pathway part 2 – animal characteristics given that an infected dairy herd gives test positive result.

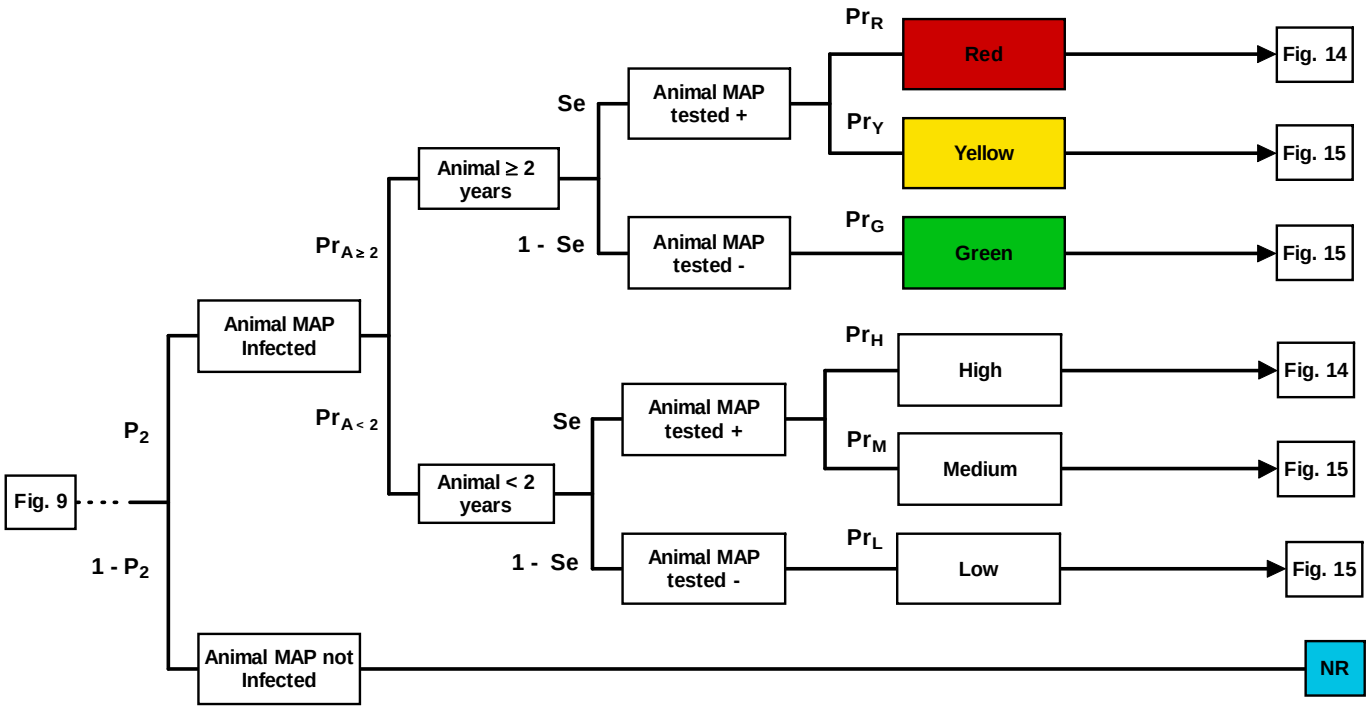

Fig. 11 – Muscle risk model pathway part 2 – animal characteristics given that an infected dairy herd gives test negative result.

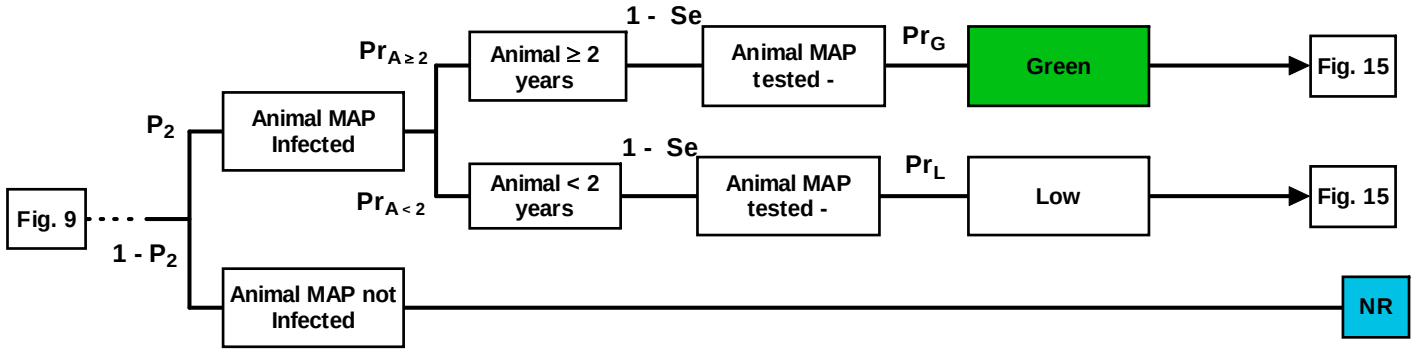

Fig. 12 – Muscle risk model pathway part 2 – animal characteristics given that an infected non-dairy herd gives test positive result.

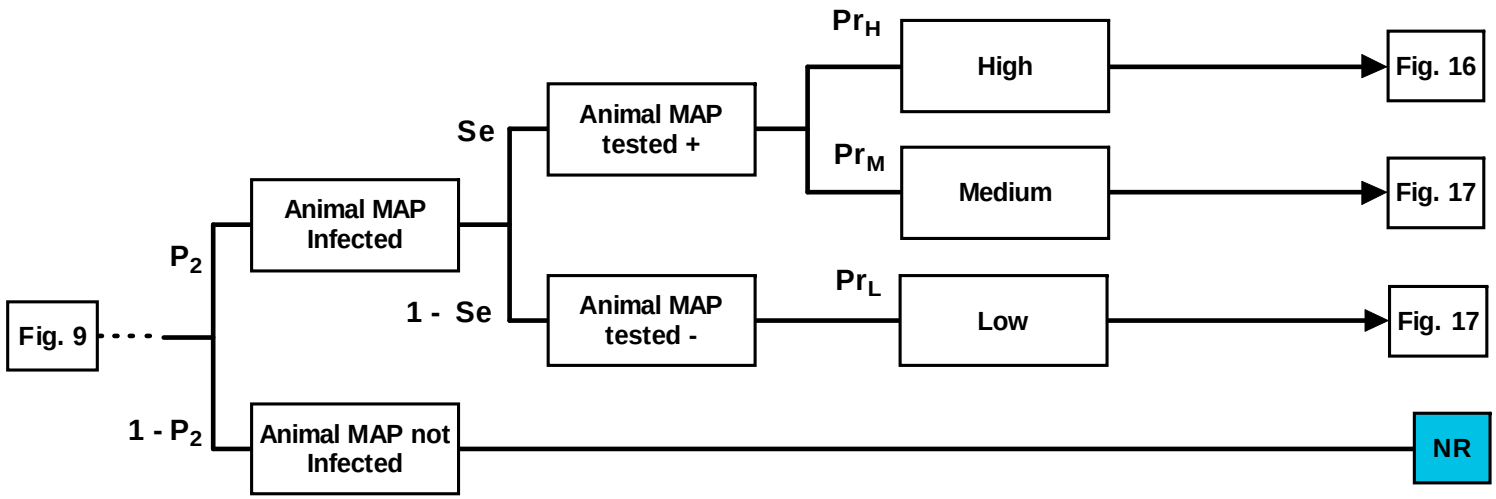

Fig. 13 – Muscle risk model pathway part 2 – animal characteristics given that an infected non-dairy herd gives test negative result.

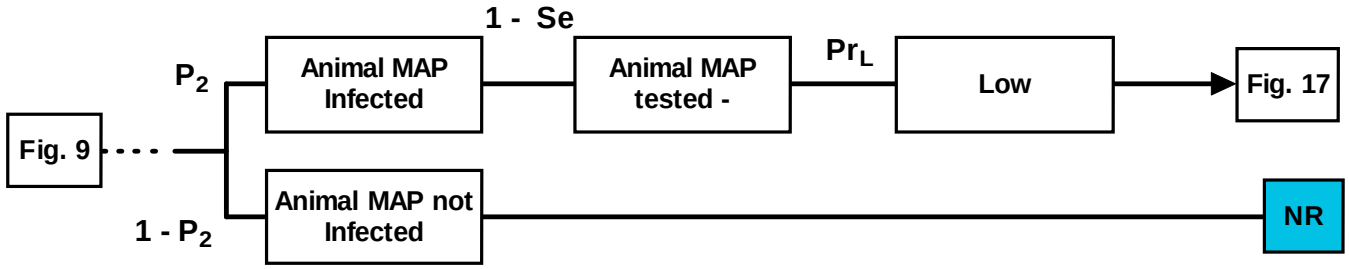

Fig. 14 – Muscle risk model pathway part 3 – animal characteristics given that a Red or High risk group animal (from dairy herd) - observable factors that could be investigated at slaughterhouse environment. It is assumed that an animal in the Red risk group or in the High risk group showing clinical signs at the *ante mortem* inspection will be rejected. The dotted line represents the lack of data to support the relation between MAP dissemination to muscle and the previous boxes in the figure.

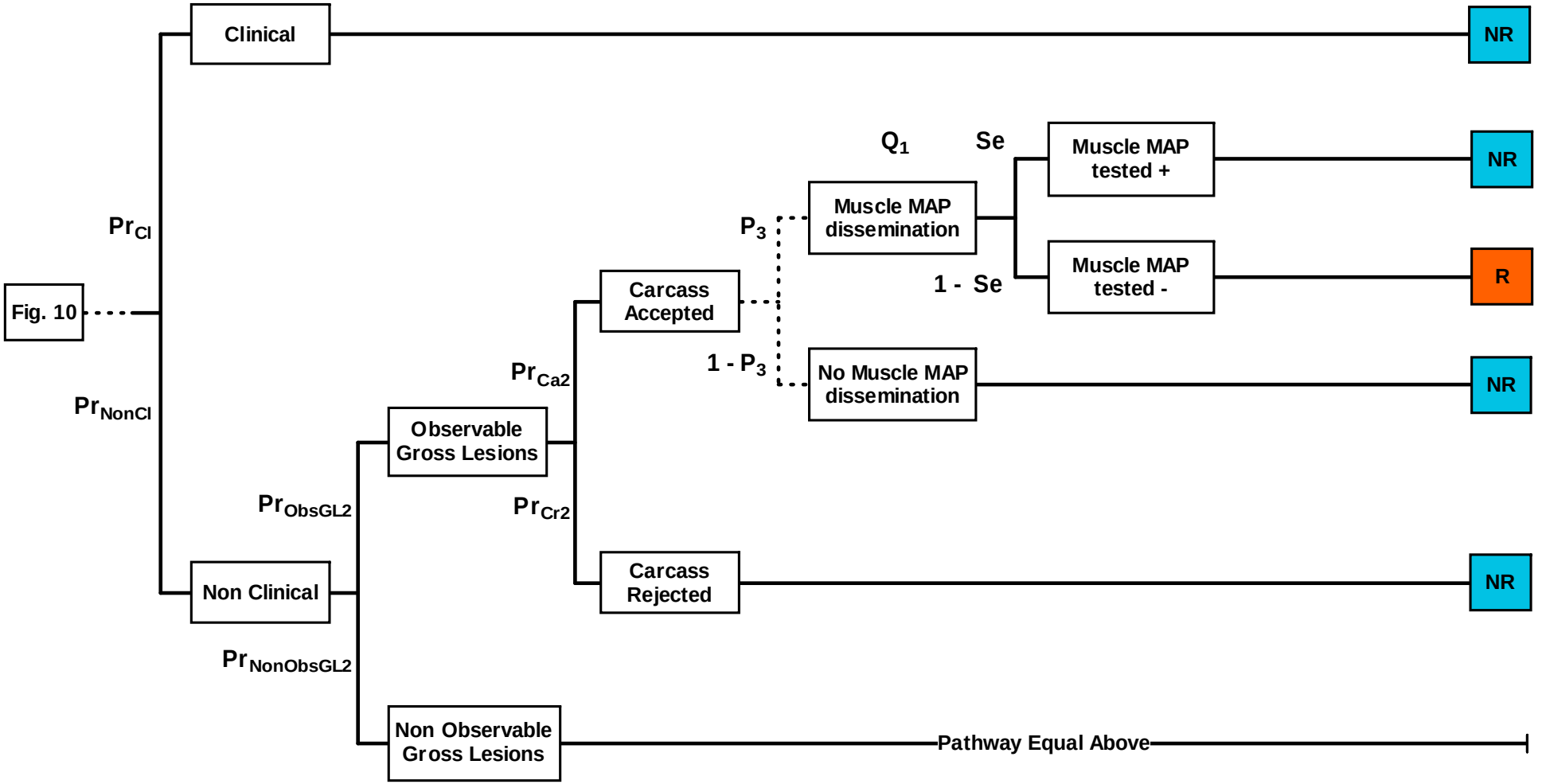

Fig. 15 – Muscle risk model pathway part 3 – animal characteristics given that a Yellow/Green or Medium/Low risk group animal (from dairy herd) - observable factors that could be investigated at slaughterhouse environment. The dotted line represents the lack of data to support the relation between MAP dissemination to muscle and the previous boxes in the figure.

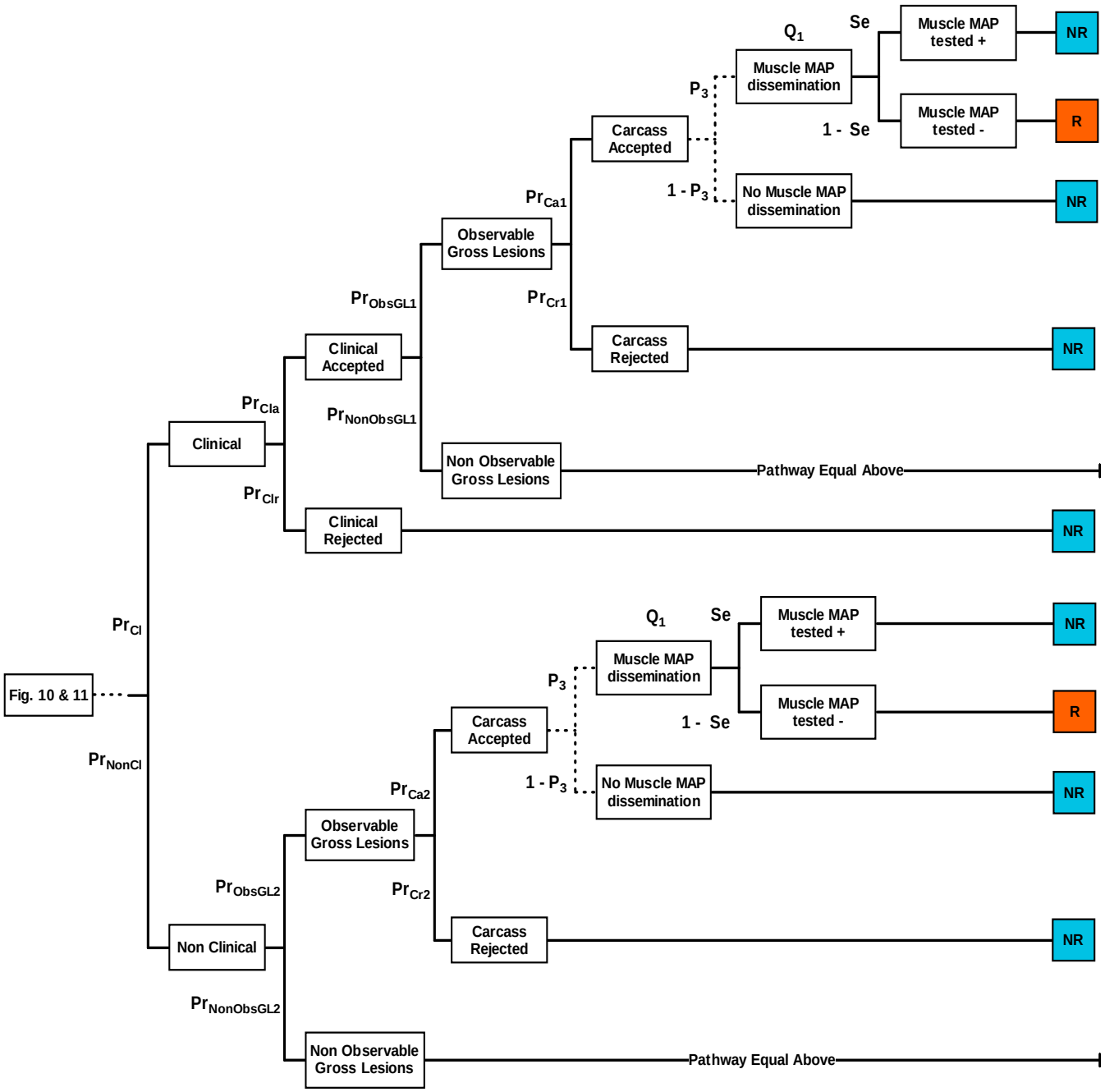

Fig. 16 – Muscle risk model pathway part 3 – animal characteristics given that a High risk group animal (from non-dairy herd) - observable factors that could be investigated at slaughterhouse environment. It is assumed that an animal in the High risk group showing clinical signs at the *ante mortem* inspection will be rejected. The dotted line represents the lack of data to support the relation between MAP dissemination to muscle and the previous boxes in the figure.

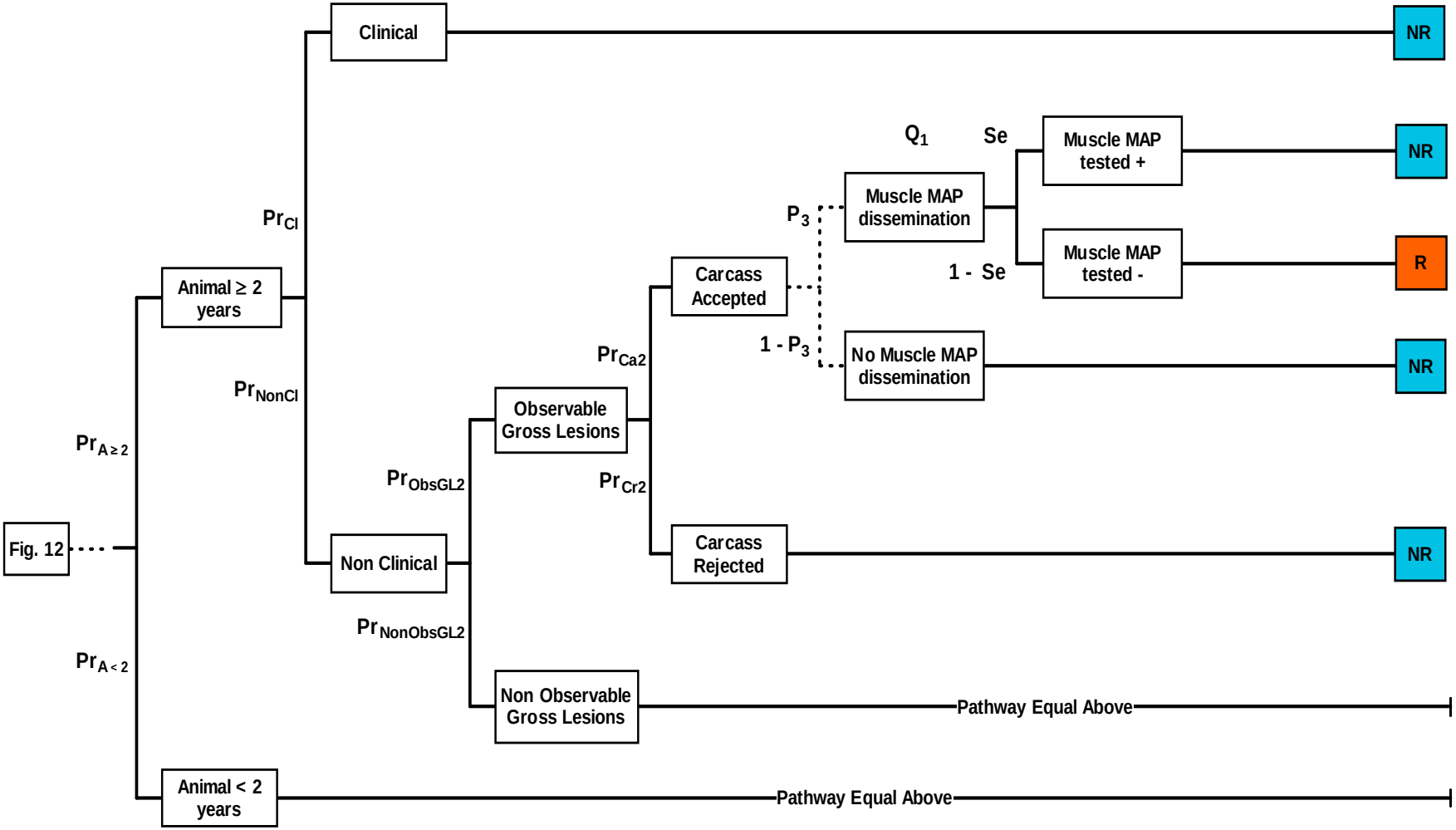

Fig. 17 – Muscle risk model pathway part 3 – animal characteristics given that a Medium/Low risk group animal (from non-dairy herd) - observable factors that could be investigated at slaughterhouse environment. The dotted line represents the lack of data to support the relation between MAP dissemination to muscle and the previous boxes in the figure.

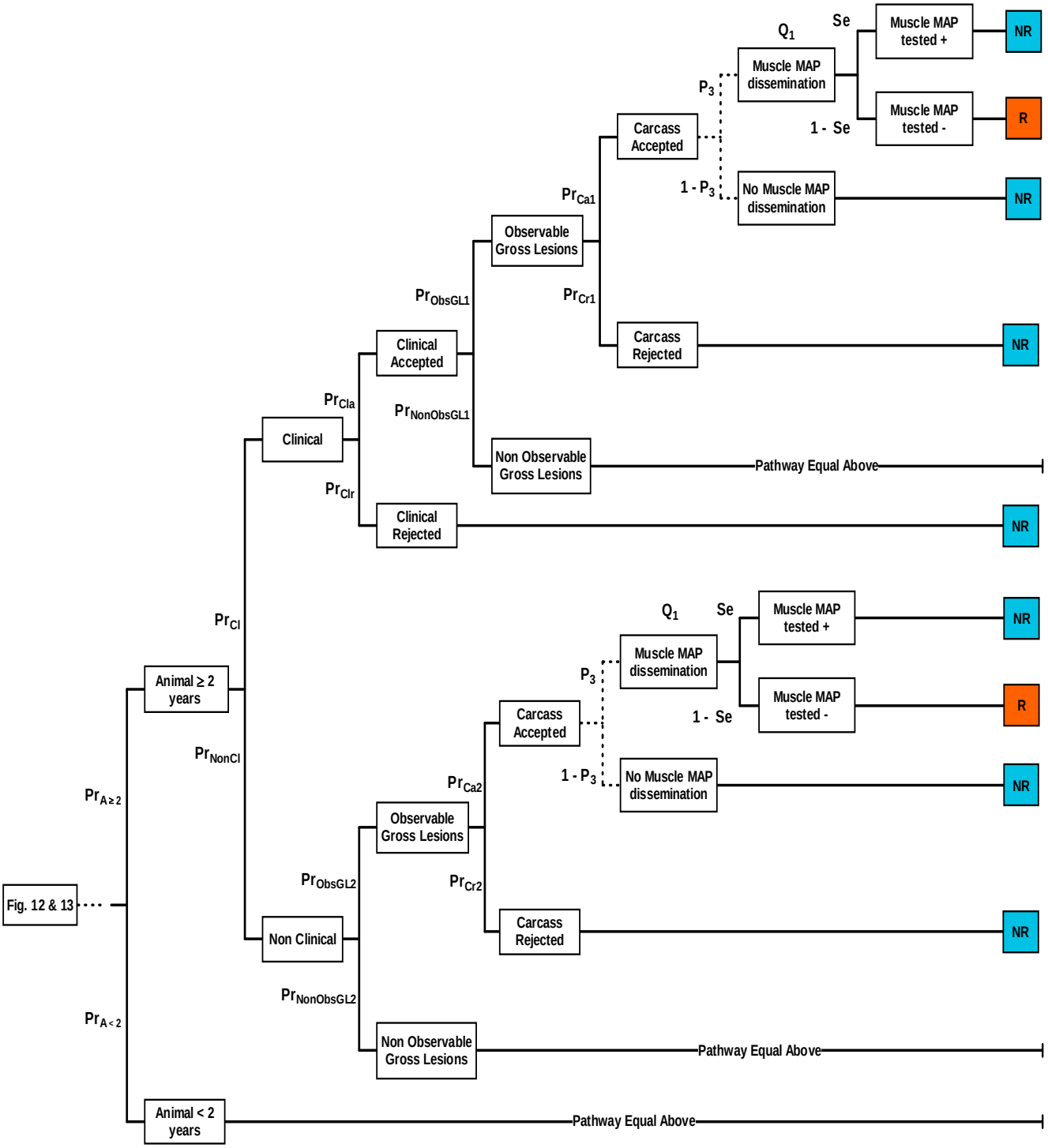

Table 8 – Muscle risk model pathway legend.

Legend	Description
Herds in ParaTB Programme	Due to thoroughly Animal testing in a ParaTB Programme, information about Herd Infection and Test Status should be available for herds in the ParaTB Programme. The ParaTB prevalence will also be influence by the existence of the control programme.
Herds not in ParaTB Programme	
Dairy Herd / Non Dairy Herd	Considering differences in the animal management system the further steps should be assessed separately for Dairy/Non Dairy herds.
Herd MAP infected	Herd Infection Status. In a Non Infected Herd is considered that all animals are Not Infected and consequently there is No Risk of MAP occurring in muscle.
Herd MAP not infected	
Herd MAP tested +	Herd Test Status. An Infected Herd can be Test Positive or Negative regarding the Test Sensitivity. In a Non Infected Herd there is No Risk regardless the Herd Test result.
Herd MAP tested -	
Animal MAP Infected	Animal Infected Status. In a Non Infected Animal there is No Risk of MAP occurring in muscle.
Animal MAP not Infected	
Animal MAP tested +	Animal Test Status. An Infected Animal can be Test Positive or Negative regarding the Test Sensitivity. In a Non Infected Animal there is No Risk regardless the Animal Test result.
Animal MAP tested -	
Red / High	Animal test results characterization representing the different animal infection stages. Colour scheme is used in the Danish ParaTB Programme. The characteristics of Danish milk testing scheme and risk group's colours will be used to differentiate risk groups in cattle ≥ 2 years old from Dairy herds. Another hypothetical differentiation in risk groups (High/Medium/Low) will be used for non dairy cattle and dairy cattle < 2 years old (not providing milk). These risk groups could be assessed with other diagnostic test more suitable to these specific animals
Yellow / Medium	
Green / Low	
Animals ≥ 2 years / < 2 years	Since MAP infection is age-related the risk should be assessed by age groups (Animals with or older than 2 years and animals younger than 2 years). On the other hand, the quantities of beef produced by these age groups are different so it will be relevant to know which group poses a higher risk.
Clinical / Non Clinical	If a relation between MAP dissemination to muscle and clinical signs if found, risk should be assessed separately regarding expression/non expression of clinical signs.
Clinical Accepted / Rejected	Considering a relation between MAP dissemination to muscle and clinical signs, and the existence of specific clinical signs representing MAP dissemination to muscle, animals could be rejected at the *ante mortem* inspection at the slaughterhouse and be considered as No Risk.
Observable Gross Lesions	If a relation between MAP dissemination to muscle and *post mortem* observable gross lesions if found, risk should be assessed separately regarding the existence of observable/non observable gross lesions.
Non Observable Gross Lesions	
Carcass Accepted / Rejected	Considering a relation between MAP dissemination to muscle and observable gross lesions, and the existence of specific gross lesions representing MAP dissemination to muscle, carcasses could be rejected at the *post mortem* inspection at the slaughterhouse and be considered as No Risk.
MAP disseminated to Muscle	MAP occurrence in Muscle by MAP dissemination to muscle in an infected animal. This MAP occurrence should be related with all previous steps in the pathway. If MAP has not disseminated to muscle there is No risk regardless the Muscle test result.
MAP not disseminated to Muscle	
Muscle MAP tested +	Muscle Test Status for the MAP occurrence. Muscle where MAP dissemination has occurred can be MAP Test Positive or Negative regarding the Test Sensitivity. A muscle MAP tested positive may be considered as No Risk if this muscle is discarded not entering the food chain.
Muscle MAP tested -	
Pathway Equal Above	Above pathway repeats.
NR	No Risk
R	Risk

Table 9 - Muscle risk model pathway description: list of variables and data availability.

Variable	Name	Comments	Availability
P_1	Probability of a herd be MAP Infected	Between-herd true prevalence for the herds in the ParaTB Programme. It should be available for the Dairy and Non Dairy herds. The measures implemented due to the existence of a ParaTB Programme will influence this prevalence.	Data available for Denmark (but only apparent prevalence)
$1 – P_1$	Probability of a herd be MAP not Infected		
P_2 / 1 - P_2	Probability of a herd be MAP Infected / not Infected	Within-herd true prevalence.	Data available
P_3 / 1 – P_3	Probability of MAP dissemination / non dissemination to muscle	Prevalence of MAP occurrence in Muscle. This information should be related with animal infection stage. To our knowledge this information is not currently available.	**Not available**
Q_1	Quantity of MAP occurrence in muscle by MAP dissemination to muscle	Quantity of MAP occurrence in milk by MAP dissemination to milk. This information should be related with animal infection stage.	**Not available**
$P_{T\text{-}BHTP}$ / 1- $P_{T\text{-}BHTP}$	Probability of a herd be MAP Infected / not Infected	Theoretical between-herd true prevalence for the herds not in the ParaTB Programme (could be literature-based).	Data available
$P_{T\text{-}WHTP}$ / 1- $P_{T\text{-}WHTP}$	Probability of an animal be MAP Infected / not Infected	Theoretical within-herd true prevalence for the herds not in the ParaTB Programme (could be literature-based).	Data available
$P_{T\text{-}MMD}$ / 1- $P_{T\text{-}MMD}$	Probability of MAP dissemination / Non dissemination to muscle	Theoretical prevalence of MAP occurrence in muscle by MAP dissemination to muscle (could be literature-based).	**Not available**
Q_T	Quantity of MAP occurrence in muscle by MAP dissemination to muscle	Theoretical quantity of MAP occurrence in muscle by MAP dissemination to muscle (could be literature-based).	**Not available**
HSe / (1 – HSe)	Herd sensitivity of the test used	An Infected Herd can be considered test positive or negative regarding the test sensitivity.	Data available
Se / (1 – Se)	Sensitivity of the test used	An Infected Animal or Muscle can be considered test positive or negative regarding the test sensitivity.	Data available
Pr_{inPr}	Proportion of herds in the ParaTB Programme	It is important to make this differentiation because it will influence the ParaTB prevalence and the available information about infection and test status of the "herd", "animal" and "muscle".	Data available
Pr_{notPr}	Proportion of herds not in the ParaTB Programme		
Pr_D / Pr_{NonD}	Proportion of Dairy/Non Dairy herds	------	Data available
Pr_G / Pr_Y / Pr_R	Proportion of cows in the Green/Yellow/Red risk group	It is important to make this differentiation because different proportions of risk groups will influence the final MAP occurrence in Muscle. For the variables Pr_{ObsGL}/$Pr_{NonObsGL}$ and Pr_{Ca} / Pr_{Cr}, the ones with number 1 correspond to animals expressing clinical signs and variables with number 2 to animals not expressing clinical signs.	Data available
Pr_L / Pr_M / Pr_H	Proportion of cows in the Low/Medium/High risk group		
$Pr_{\geq 2\ years}$ / $Pr_{<2\ years}$	Proportion of cows ≥ 2 years / < 2 years		
Pr_{Cl} / $Pr_{Non\ Cl}$	Proportion of animals expressing/not expressing clinical signs		**Not Available**
Pr_{Cla} / Pr_{Clr}	Proportion of animals that are clinical accepted /rejected		
Pr_{ObsGL}/$Pr_{NonObsGL}$	Proportion of animals with/without observable gross lesions		
Pr_{Ca} / Pr_{Cr}	Proportion of carcasses accepted / rejected		

4.4.1 Data availability and risk description

Data availability

Most information about the herd and the animal characteristics should be available on the databases of the ParaTB control programme. However, in the case of the Danish ParaTB control programme not all information is available with the detail proposed by this risk model pathway (also because it was the control programme was designed for dairy cattle only):

- Only herds in the ParaTB programme have information about the herd and animal infection and test status. This information for herds not in the ParaTB programme is not available (theoretical literature-based values could be considered).
- The allocation of animals into different risk groups is based upon animal's milk antibody profile. To our knowledge, the mechanism of MAP dissemination to muscle is not understood so the relation between the degree and quantity of MAP dissemination to muscle and the antibody response is unknown. Then there is no data available for the different probabilities and quantity of MAP excretion on muscle by different risk groups.
- The characteristics of Danish milk testing scheme and risk group's colours were used to differentiate risk groups in cattle ≥ 2 years old from Dairy herds. Another hypothetical differentiation in risk groups (High/Medium/Low) was used for non dairy cattle and dairy cattle < 2 years old (not providing milk). These risk groups could be assessed with other diagnostic test more suitable to these specific animals.
- The relation between the degree and quantity of MAP dissemination to muscle and age and other observable factors (clinical signs and gross lesions) at slaughterhouse environment is unknown. Then there is no data available for the different probabilities and quantity of MAP excretion on muscle by these different risk groups (e.g. by animals demonstrating clinical signs, being clinical accepted, demonstrating observable gross lesions and the carcass be accepted).

Risk description

In the context of this risk model pathway is assumed that don't exist risk to MAP entering the food chain when herds, animals and muscle are considered Not Infected, when an infected animal or an animal from Red/High risk group and showing clinical signs is rejected at *ante mortem* inspection, when the carcass is rejected at the *post mortem* inspection, or when is considered that has occurred MAP dissemination to muscle, it was test positive and rejected not entering the food chain. There is risk of MAP entering the food chain in the specific case of infected herds not in the ParaTB Programme (because there are infected animals where MAP dissemination to muscle may have occurred and this information is not available) and when MAP dissemination to muscle has occurred and gave test negative result.

The MAP quantities in faeces and the probability of faecal contamination at the slaughter line are important aspects related with the hygiene procedures but are out of the scope of this work. However, in order to evaluate the MAP added to muscle by faecal contamination two values must be known: the quantity of faeces that contaminates muscle tissues at the slaughter line and the quantity of MAP in this amount of faeces regarding their origin of contamination. The quantity of MAP contamination by faecal contamination may also be related with the proportion animals of different risk groups that are slaughter together, because the existence or absence of a large proportion of highly shedders or highly contaminated animals in the slaughter line will influence the quantity of MAP in faeces that may contaminate muscle tissues.

Depending on the objectives of the risk assessment and of the scope of the risk question, the units to each parameter should be assigned. For example, the evaluation of each probability and MAP quantity could be made per kilogram of muscle tissue at the end of the slaughter line.

As an example, the final probability for MAP occurrence on muscle MAP test negative from infected cows older than 2 years, with clinical signs that have been accepted in the *ante mortem* inspection, with gross lesions that have been accepted in the *post mortem* inspection, characterized as being from the Red risk group, from an infected and test positive dairy herd in the ParaTB programme is the product between:

- proportion of herds in the ParaTB programme;
- proportion of dairy herds;
- the probability of the herd be MAP infected;
- the herd sensitivity of the test;
- the probability of the animal be MAP infected;
- proportion of animals older than 2 years;
- the sensitivity of the test used;
- the proportion of cows in the Red risk group;
- proportion of animals with clinical signs;
- proportion of animals with clinical signs accepted at the *ante mortem* inspection;
- proportion of carcasses with observable gross lesions;
- proportion of carcasses with observable gross lesions accepted at the *post mortem* inspection;
- probability of MAP occurrence on muscle tissues from animals with the mentioned characteristics;
- (1 – sensitivity) of the test used.

In this scenario the final risk would also consider the quantity of MAP occurrence in muscle by MAP dissemination to muscle in cows with the characteristics mentioned before.

4.5 Conclusions

As shown in this dissertation a risk assessment conceptual model was required in order to assess the current knowledge on this topic. This was made in the framework of the Danish Paratuberculosis control programme despite Paratuberculosis has not yet been declared as a zoonosis.

An exhaustive literature search was made to assess one point of the risk model pathway – MAP distribution on tissues. MAP presence on muscle by tissue culture was only investigated by two studies; in milk by ten studies. Most of the studies focused their attention on the gastro-intestinal tract. However MAP was isolated on several other tissues. Besides MAP isolation sites, scarce information exists about the mechanisms of MAP dissemination on tissues, when this dissemination occurs and its relation with other parameters as serum and milk antibodies, cellular immune response, MAP excretion on milk and faeces, gross and histopathologic lesions and clinical signs.
From our study a merely indicative overall prevalence of MAP tissue contamination was assessed as 10.2% [9.8% - 10.6%], a milk prevalence of 4.7% [3.9% – 5.7%] and muscle prevalence of 4.8% [2.4% - 9.2%]. These results could not be used as the likelihood MAP occurrence in these tissues due to the results limitations discussed previously.

Risk assessment is a tool that enables the analysis of a problem in a systematic, transparent and reliable way. In order to assess the probability and the quantity of MAP occurrence on muscle and milk all the pathways that may lead to MAP presence on the milk at herd level or on muscle tissues at the end of the slaughter line should be considered. In this work, a risk model pathway to analyze MAP occurrence on unprocessed beef and milk is proposed. When assembling the necessary data for risk evaluation, knowledge gaps concerning MAP distribution on milk and beef were found.
Without the understanding of the relation between MAP probability/quantity and specific characteristics of different risk groups, the risk cannot be assessed properly. Therefore measures to reduce risk cannot be taken on specific animals or at specific points of the process.
The main limitations and drawbacks of proposed risk model pathways are:

- Lack of detailed information for herds not in the ParaTB Programme;
- Current difficulty in certifying Herds, Animals or Milk/Muscle “Not MAP Infected” due to available diagnostic tools;
- Low sensitivity of the available diagnostic tools and the time-dependent animal responses;

- Lack of information regarding the relation between MAP occurrence in muscle and animal infection stage, age risk groups, existence/absence of clinical signs and clinical signs that may lead to *ante mortem* animal rejection, existence/absence of observable gross lesions in clinical/non clinical animals and gross lesions that may lead to *post mortem* carcass rejection.

Regarding the risk model pathways, MAP contamination by faeces was out of the scope of the work. However, it is an aspect that should not be neglected. The impact of each source of MAP occurrence on unprocessed beef and milk (lymphatic/bacteraemia/faecal) should also be evaluated to implement specific mitigation measures.

The characterization of the occurrence and the quantity of MAP in tissues and the assessment of the probability/quantity of MAP occurrence on beef and milk may have an extensive range of applications such as:

- Indication of the best sample sites to detect MAP infection by tissue culture;
- Evaluation of the impact that specific food processes have on MAP survival (freezing, pasteurization, cooking);
- Recommendations to meat inspectors to reject/approve animals/carcasses to assure food safety;
- Recommendations to farmers regarding milk collection from high risk animals, milk hygiene and herd management to reduce the risk of MAP occurrence on animal products;
- Recommendations to industry about the use of high and low risk animal products in human food;
- Recommendations to Veterinary Authorities regarding possible changes in animal health programmes considering the perspective of MAP infection as a possible hazard to food safety.

In conclusion, a list of studies, where MAP was isolated by tissue culture, is available in this dissertation and may be useful to other authors looking for this gathered information. Some current knowledge gaps and lacks of information were pointed out. Regarding the risk assessment a detailed list of data necessary to make possible the evaluation of MAP sources to consumers is provided by this study. Milk and beef risk model pathways have also been developed and may serve risk assessors with a basis for their work.
Further studies would be interesting to make available the mentioned knowledge gaps and to develop and improve diagnostic tests for direct MAP detection on specific tissues.

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6 Annex

6.1 Annex 1 - Tissue culture results recorded from the reviewed studies.

Annex 1 – Tissue culture results recorded from the reviewed studies as well as the clinical status, husbandry type and age range of the studied animals. The results are grouped by tissue sampled and by reference alphabetic order. Summaries from each tissue were made. The prevalence and 95% Confidence intervals are shown. Annex 1 is composed by 16 tables.[18]

[18] Sum. – Summary; Nd – No data; cont. – continuation; Husb. – Husbandry; Muc. – Mucosa; Af – Affected animals; As – Asymptomatic animals; B – Beef; D – Dairy; LN – Lymph node; Prev. – Prevalence; 95% CI – 95% Confidence interval; <2y – younger than 2 years old; ≥2y – with or older than 2 years old; GI – Gastro-intestinal; Gl. – gland; Sup.Mam. – Supramammary; Mam.gl. – Mammary gland; In the study from Brady et al (2008) they considered abdominal organs as spleen, kidney, liver, hepatic lymph node, and thoracic organs as lungs, heart, bronchial and mediastinal lymph node.

Annex 1.1

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Abomasum muc.	2	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	2	7	Both	D	≥2y				(Taylor, 1953)
Sum. Abomasum muc.	**4**	**44**				**9.1%**	**3.6%**	**21.2%**	
Pylorus muc.	6	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
Sum. Pylorus muc.	**6**	**37**				**16.2%**	**7.7%**	**31.1%**	
Duodenum muc.	3	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	15	Both	D	≥2y				(Huda & Jensen, 2003)
	0	29	As	D	≥2y				(McDonald, Ridge, Hope & Condron, 1999)
	6	21	As	D	<2y				(Payne & Rankin, 1961b)
	1	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	3	3	Both	D	<2y				(Rankin, 1958)
	1	3	Both	D	≥2y				(Rankin, 1958)
	1	5	As	D	≥2y				(Rankin, 1961a)
	5	9	Both	D	≥2y				(Rankin, 1961b)
Sum. Duodenum muc.	**20**	**115**				**17.4%**	**11.5%**	**25.3%**	
Jejunum muc.	3	5	Both	B	≥2y				(Alonso-Hearn et al., 2009)
	18	42	Both	D	≥2y				(Alonso-Hearn et al., 2009)
	16	40	Af	D	≥2y				(Antognoli et al., 2008)
	3	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	2	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	4	15	Both	D	≥2y				(Huda & Jensen, 2003)
	1	1	Af	D	≥2y				(Larsen et al., 1981)
	2	29	As	D	≥2y				(McDonald, Ridge, Hope & Condron, 1999)
	4	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	1	8	As	D	<2y				(Payne & Rankin, 1961a)
Sum. Jejunum muc.	**54**	**183**				**29.5%**	**23.4%**	**36.5%**	

Annex 1.2

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Ileum muc.	18	40	Af	D	≥2y				(Antognoli et al., 2008)
	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	3	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	18	20	Both	Nd	Nd				(Brady et al., 2008)
	3	100	As	D	≥2y				(Chiodini & Vankruiningen, 1986)
	6	15	Both	D	≥2y				(Huda & Jensen, 2003)
	1	1	Af	D	≥2y				(Larsen et al., 1981)
	6	29	As	D	≥2y				(McDonald, Ridge, Hope & Condron, 1999)
	85	984	As	D	≥2y				(McKenna et al., 2004)
	4	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	3	3	Both	D	<2y				(Rankin, 1958)
	1	3	Both	D	≥2y				(Rankin, 1958)
	1	5	As	D	≥2y				(Rankin, 1961a)
	7	9	Both	D	≥2y				(Rankin, 1961b)
	4	12	As	B	<2y				(Stabel, Palmer & Whitlock, 2003)
Sum. Ileum muc.	**160**	**1272**				**12.6%**	**10.9%**	**14.5%**	
Cecum muc.	3	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	5	15	Both	D	≥2y				(Huda & Jensen, 2003)
	1	29	As	D	≥2y				(McDonald, Ridge, Hope & Condron, 1999)
	6	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	3	3	Both	D	<2y				(Rankin, 1958)
	2	3	Both	D	≥2y				(Rankin, 1958)
	1	5	As	D	≥2y				(Rankin, 1961a)
	6	9	Both	D	≥2y				(Rankin, 1961b)
	13	16	As	D	≥2y				(Whitlock et al., 1997)
Sum. Cecum muc.	**40**	**121**				**33.1%**	**25.3%**	**41.8%**	

Annex 1.3

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Ileo-cecal valve muc.	2	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	3	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	5	100	As	D	≥2y				(Chiodini & Vankruiningen, 1986)
	4	15	Both	D	≥2y				(Huda & Jensen, 2003)
	4	7	Af	D	≥2y				(Koenig et al., 1993)
	58	148	Both	D	≥2y				(Kopecky, Larsen & Merkal, 1967)
	5	29	As	D	≥2y				(McDonald, Ridge, Hope & Condron, 1999)
	10	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	1	12	As	B	<2y				(Stabel, Palmer & Whitlock, 2003)
	27	35	As	D	≥2y				(Whitlock et al., 1997)
Sum. Ileo-cecal valve muc.	**119**	**397**				**30.0%**	**25.7%**	**34.7%**	
Colon muc.	2	29	As	D	≥2y				(McDonald, Ridge, Hope & Condron, 1999)
	8	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	1	8	As	D	<2y				(Payne & Rankin, 1961a)
	8	12	As	B	<2y				(Stabel, Palmer & Whitlock, 2003)
	13	18	As	D	≥2y				(Whitlock et al., 1997)
Sum. Colon muc.	**32**	**96**				**33.3%**	**24.7%**	**43.2%**	
Rectum muc.	4	15	Both	D	≥2y				(Huda & Jensen, 2003)
	0	29	As	D	≥2y				(McDonald, Ridge, Hope & Condron, 1999)
	3	21	As	D	<2y				(Payne & Rankin, 1961b)
	1	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	1	3	Both	D	≥2y				(Rankin, 1958)
	3	3	Both	D	<2y				(Rankin, 1958)
	1	5	As	D	≥2y				(Rankin, 1961a)
	5	8	Both	D	≥2y				(Rankin, 1961b)
Sum. Rectum muc.	**18**	**100**				**18.0%**	**11.7%**	**26.7%**	

Annex 1.4

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Ileum + Cecum muc.	13	20	Af	D	<2y	65.0%	43.3%	81.9%	(Thorel, Pardon, Irgens, Marly & Lechopier, 1984)
Ileum + Ileocecal-valve muc.	5	5	Both	B	≥2y				(Alonso-Hearn et al., 2009)
	19	42	Both	D	≥2y				(Alonso-Hearn et al., 2009)
Small Intestine	15	19	Both	Nd	Nd				(Brady et al., 2008)
	3	5	As	D	<2y				(Larsen et al., 1975)
	1	3	As	D	≥2y				(Larsen et al., 1975)
	4	4	As	D	<2y				(Saxegaard, 1990)
	19	23	Both	D	≥2y				(Taylor, 1953)
Large Intestine	13	20	Both	Nd	Nd				(Brady et al., 2008)
	13	21	Both	D	≥2y				(Taylor, 1953)
Intestinal Mucosa	26	26	As	D	<2y				(Gilmour, Nisbet & Brotherston, 1965)
Sum. Intestinal Mucosa	**584**	**2553**				**22.9%**	**21.3%**	**24.5%**	

Annex 1.5

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Duodenal LN	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	3	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	1	15	Both	D	≥2y				(Huda & Jensen, 2003)
Sum. Duodenal LN	**4**	**29**				**13.8%**	**5.5%**	**30.6%**	
Jejunal LN	4	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	3	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	5	15	Both	D	≥2y				(Huda & Jensen, 2003)
	1	1	Af	D	≥2y				(Larsen et al., 1981)
Sum. Jejunal LN	**13**	**30**				**43.3%**	**27.4%**	**60.8%**	
Jejunal Peyer's Patches	5	18	As	D	<2y	27.8%	12.5%	50.9%	(Beard et al., 2001)
Ileal LN	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	3	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	4	15	Both	D	≥2y				(Huda & Jensen, 2003)
	1	1	Af	D	≥2y				(Larsen et al., 1981)
	6	12	As	B	<2y				(Stabel, Palmer & Whitlock, 2003)
Sum. Ileal LN	**14**	**42**				**33.3%**	**21.0%**	**48.4%**	

Annex 1.6

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Ileocecal LN	17	40	Af	D	≥2y				(Antognoli et al., 2008)
	16	18	Both	Nd	Nd				(Brady et al., 2008)
	3	15	Both	D	≥2y				(Huda & Jensen, 2003)
	2	448	As	D	<2y				(Jorgensen, 1965)
	15	662	As	D	≥2y				(Jorgensen, 1965)
	5	6	Both	D	≥2y				(Koenig et al., 1993)
	0	29	As	D	≥2y				(McDonald, Ridge, Hope & Condron, 1999)
	110	984	As	D	≥2y				(McKenna et al., 2004)
	11	1191	As	Both	≥2y				(Merkal, Whipple, Sacks & Snyder, 1987)
	81	2827	As	D	≥2y				(Merkal, Whipple, Sacks & Snyder, 1987)
	27	3522	As	B	≥2y				(Merkal, Whipple, Sacks & Snyder, 1987)
	12	21	As	D	<2y				(Payne & Rankin, 1961b)
	5	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	2	3	Both	D	<2y				(Rankin, 1958)
	2	3	Both	D	≥2y				(Rankin, 1958)
	3	5	As	D	≥2y				(Rankin, 1961a)
	8	9	Both	D	≥2y				(Rankin, 1961b)
	9	350	Af	B	≥2y				(Rossiter & Henning, 2001)
	65	189	Af	D	≥2y				(Rossiter & Henning, 2001)
	34	407	Both	D	≥2y				(Seitz et al., 1989)
	9	12	As	B	<2y				(Stabel, Palmer & Whitlock, 2003)
Sum. Ileocecal LN	**436**	**10757**				**4.1%**	**3.7%**	**4.4%**	
Cecal LN	8	100	As	D	≥2y	8.0%	4.1%	15.0%	(Chiodini & Vankruiningen, 1986)
Colic LN	8	12	As	B	<2y	66.7%	39.1%	86.2%	(Stabel, Palmer & Whitlock, 2003)

Annex 1.7

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Mesenteric LN	16	40	Af	D	≥2y				(Antognoli et al., 2008)
	8	18	As	D	<2y				(Beard et al., 2001)
	16	20	Both	Nd	Nd				(Brady et al., 2008)
	14	26	As	D	<2y				(Gilmour, Nisbet & Brotherston, 1965)
	3	5	As	D	<2y				(Larsen et al., 1975)
	1	3	As	D	≥2y				(Larsen et al., 1975)
	11	21	As	D	<2y				(Payne & Rankin, 1961b)
	5	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	2	3	Both	D	≥2y				(Rankin, 1958)
	2	3	Both	D	<2y				(Rankin, 1958)
	1	5	As	D	≥2y				(Rankin, 1961a)
	6	9	Both	D	≥2y				(Rankin, 1961b)
	4	4	As	D	<2y				(Saxegaard, 1990)
	51	400	As	Both	≥2y				(Smith, 1954)
	20	23	Both	D	≥2y				(Taylor, 1953)
	21	21	Af	D	<2y				(Thorel, Pardon, Irgens, Marly & Lechopier, 1984)
Sum. Mesenteric LN	**181**	**617**				**29.3%**	**25.9%**	**33.0%**	
Ileocecal + Jejunal LN	4	5	Both	B	≥2y				(Alonso-Hearn et al., 2009)
	19	42	Both	D	≥2y				(Alonso-Hearn et al., 2009)
Sum. GI Lymphoid Tissue	**692**	**11652**				**5.9%**	**5.5%**	**6.4%**	
Duodenum + LN	5	12	As	B	<2y				(Stabel, Palmer & Whitlock, 2003)
Jejunum + LN	8	12	As	B	<2y				(Stabel, Palmer & Whitlock, 2003)
Ileum muc + Ileocecal LN	22	400	As	Both	≥2y				(McNab, Meek, Duncan, Martin & Vandreumel, 1991)
GI tract	198	497	As	D	≥2y				(Pavlik et al., 2000)
	9	45	As	B	≥2y				(Pavlik et al., 2000)
	11	69	As	Both	≥2y				(Pavlik et al., 2000)
Sum. GI tract	**1529**	**15240**				**10.0%**	**9.6%**	**10.5%**	

Annex 1.8

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Mandibular LN	1	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	5	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
Sum. Mandibular LN	**6**	**51**				**11.8%**	**5.5%**	**23.4%**	
Submaxilar LN	3	3	Both	D	<2y				(Rankin, 1958)
	0	2	Both	D	≥2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	4	8	Both	D	≥2y				(Rankin, 1961b)
	11	23	Both	D	≥2y				(Taylor, 1953)
Sum. Submaxilar LN	**18**	**41**				**43.9%**	**29.9%**	**59.0%**	
Parotid LN	0	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
Sum. Parotid LN	**0**	**37**				**0.0%**	**0.0%**	**9.4%**	
Suprapharyngeal LN	12	21	As	D	<2y				(Payne & Rankin, 1961b)
	6	8	As	D	<2y				(Payne & Rankin, 1961a)
	1	8	As	D	≥2y				(Payne & Rankin, 1961a)
Sum. Suprapharyngeal LN	**19**	**37**				**51.4%**	**35.9%**	**66.6%**	
Retropharyngeal LN	4	40	Af	D	≥2y				(Antognoli et al., 2008)
	0	26	As	D	<2y				(Gilmour, Nisbet & Brotherston, 1965)
	0	15	Both	D	≥2y				(Huda & Jensen, 2003)
	1	2	Both	D	≥2y				(Rankin, 1958)
	3	3	Both	D	<2y				(Rankin, 1958)
	3	5	As	D	≥2y				(Rankin, 1961a)
	8	8	Both	D	≥2y				(Rankin, 1961b)
	15	23	Both	D	≥2y				(Taylor, 1953)
Sum. Retropharyngeal LN	**34**	**122**				**27.9%**	**20.7%**	**36.4%**	

Annex 1.9

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Tonsil	2	100	As	D	≥2y				(Chiodini & Vankruiningen, 1986)
	0	26	As	D	<2y				(Gilmour, Nisbet & Brotherston, 1965)
	0	15	Both	D	≥2y				(Huda & Jensen, 2003)
	10	21	As	D	<2y				(Payne & Rankin, 1961b)
	2	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
Sum. Tonsil	**14**	**178**				**7.9%**	**4.7%**	**12.8%**	
Head LN	11	18	Both	Nd	Nd				(Brady et al., 2008)
	16	22	As	D	≥2y				(Whitlock et al., 1997)
Sum. Head Lymphoid tissue	**118**	**506**				**23.3%**	**19.8%**	**27.2%**	
Bronchial LN	0	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
Sum. Bronchial LN	**0**	**37**				**0.0%**	**0.0%**	**9.4%**	
Mediastinal LN	1	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	26	As	D	<2y				(Gilmour, Nisbet & Brotherston, 1965)
	0	15	Both	D	≥2y				(Huda & Jensen, 2003)
	1	1	Af	D	≥2y				(Larsen et al., 1981)
	2	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	3	3	Both	D	<2y				(Rankin, 1958)
	0	2	Both	D	≥2y				(Rankin, 1958)
	1	5	As	D	≥2y				(Rankin, 1961a)
	5	8	Both	D	≥2y				(Rankin, 1961b)
	15	23	Both	D	≥2y				(Taylor, 1953)
Sum. Mediastinal LN	**28**	**134**				**20.9%**	**14.9%**	**28.5%**	
Pulmonary LN	32	82	As	D	≥2y	39.0%	29.2%	49.8%	(Whitlock et al., 1997)
Superficial Cervical LN	6	189	Af	D	≥2y				(Rossiter & Henning, 2001)
	1	350	Af	B	≥2y				(Rossiter & Henning, 2001)
Sum. Superficial Cervical LN	**7**	**539**				**1.3%**	**0.6%**	**2.7%**	

Annex 1.10

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Hepatic LN	17	40	Af	D	≥2y				(Antognoli et al., 2008)
	1	15	Both	D	≥2y				(Huda & Jensen, 2003)
	8	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	1	8	As	D	<2y				(Payne & Rankin, 1961a)
	1	1	Both	D	≥2y				(Rankin, 1958)
	1	5	As	D	≥2y				(Rankin, 1961a)
	6	8	Both	D	≥2y				(Rankin, 1961b)
	2	12	As	B	<2y				(Stabel, Palmer & Whitlock, 2003)
	16	23	Both	D	≥2y				(Taylor, 1953)
	41	80	As	D	≥2y				(Whitlock et al., 1997)
Sum. Hepatic LN	**94**	**221**				**42.5%**	**36.2%**	**49.1%**	
Iliac LN	2	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	2	Both	D	≥2y				(Rankin, 1958)
	3	3	Both	D	<2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	3	8	Both	D	≥2y				(Rankin, 1961b)
	2	12	As	B	<2y				(Stabel, Palmer & Whitlock, 2003)
	1	2	Both	D	≥2y				(Taylor, 1953)
Sum. Iliac LN	**11**	**69**				**15.9%**	**9.1%**	**26.3%**	
Popliteal LN	3	40	Af	D	≥2y				(Antognoli et al., 2008)
	0	26	As	D	<2y				(Gilmour, Nisbet & Brotherston, 1965)
	4	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	2	Both	D	≥2y				(Rankin, 1958)
	3	3	Both	D	<2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	2	8	Both	D	≥2y				(Rankin, 1961b)
	1	350	Af	B	≥2y				(Rossiter & Henning, 2001)
	6	189	Af	D	≥2y				(Rossiter & Henning, 2001)
Sum. Popliteal LN	**19**	**660**				**2.9%**	**1.9%**	**4.5%**	

Annex 1.11

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Precrural LN	6	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
Sum. Precrural LN	**6**	**37**				**16.2%**	**7.7%**	**31.1%**	
Prescapular LN	1	40	Af	D	≥2y				(Antognoli et al., 2008)
	0	26	As	D	<2y				(Gilmour, Nisbet & Brotherston, 1965)
	5	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
Prescapular LN (cont.)	0	2	Both	D	≥2y				(Rankin, 1958)
	3	3	Both	D	<2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	3	8	Both	D	≥2y				(Rankin, 1961b)
	6	6	Both	D	≥2y				(Taylor, 1953)
Sum. Prescapular LN	**18**	**127**				**14.2%**	**9.2%**	**21.3%**	
Supramammary LN	6	40	Af	D	≥2y				(Antognoli et al., 2008)
	0	34	Af	D	≥2y				(Doyle, 1954)
	0	15	Both	D	≥2y				(Huda & Jensen, 2003)
	0	2	Both	D	≥2y				(Rankin, 1958)
	3	3	Both	D	<2y				(Rankin, 1958)
	1	5	As	D	≥2y				(Rankin, 1961a)
	3	7	Both	D	≥2y				(Rankin, 1961b)
	22	81	As	D	≥2y				(Sweeney et al., 1992b)
	1	4	Af	D	≥2y				(Taylor et al., 1981)
	16	60	As	D	≥2y				(Whitlock et al., 1997)
Sum. Supramammary LN	**52**	**251**				**20.7%**	**16.2%**	**26.2%**	
Prescapular and Popliteal LN	13	19	Both	Nd	Nd				(Brady et al., 2008)
Sum. Other LN	**398**	**2682**				**14.8%**	**13.5%**	**16.2%**	
Sum. All Lymphoid tissue	**1090**	**14334**				**7.6%**	**7.2%**	**8.0%**	

Annex 1.12

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Kidney	6	40	Af	D	≥2y				(Antognoli et al., 2008)
	1	1	Af	D	≥2y				(Hines et al., 1987)
	0	1	Af	D	≥2y				(Larsen et al., 1981)
	1	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	3	Both	D	≥2y				(Rankin, 1958)
	3	3	Both	D	<2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	2	8	Both	D	≥2y				(Rankin, 1961b)
Sum. Kidney	**13**	**98**				**13.3%**	**7.9%**	**21.4%**	
Liver	10	40	Af	D	≥2y				(Antognoli et al., 2008)
	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	2	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	2	100	As	D	≥2y				(Chiodini & Vankruiningen, 1986)
	0	26	As	D	<2y				(Gilmour, Nisbet & Brotherston, 1965)
	0	1	Af	D	≥2y				(Larsen et al., 1981)
	3	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	3	Both	D	≥2y				(Rankin, 1958)
	3	3	Both	D	<2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	3	8	Both	D	≥2y				(Rankin, 1961b)
	15	189	Af	D	≥2y				(Rossiter & Henning, 2001)
	1	350	Af	B	≥2y				(Rossiter & Henning, 2001)
	15	22	Both	D	≥2y				(Taylor, 1953)
Sum. Liver	**54**	**798**				**6.8%**	**5.2%**	**8.7%**	

Annex 1.13

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Lung	4	40	Af	D	≥2y				(Antognoli et al., 2008)
	1	1	Af	D	≥2y				(Larsen et al., 1981)
	1	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	3	Both	D	≥2y				(Rankin, 1958)
	2	3	Both	D	<2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	2	8	Both	D	≥2y				(Rankin, 1961b)
	10	22	Both	D	≥2y				(Taylor, 1953)
Sum. Lung	**20**	**119**				**16.8%**	**11.2%**	**24.5%**	
Spleen	1	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	1	26	As	D	<2y				(Gilmour, Nisbet & Brotherston, 1965)
	1	1	Af	D	≥2y				(Larsen et al., 1981)
	4	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	3	Both	D	≥2y				(Rankin, 1958)
	3	3	Both	D	<2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	1	8	Both	D	≥2y				(Rankin, 1961b)
	6	21	Both	D	≥2y				(Taylor, 1953)
	15	83	As	D	≥2y				(Whitlock et al., 1997)
Sum. Spleen	**32**	**201**				**15.9%**	**11.5%**	**21.6%**	

Annex 1.14

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Bulbourethral gl.	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	3	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	2	4	Af	Both	≥2y				(Larsen & Kopecky, 1970)
Sum. Bulbourethral gl.	**2**	**17**				**11.8%**	**3.3%**	**34.3%**	
Epididymis	2	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
Sum. Epididymis	**2**	**14**				**14.3%**	**4.0%**	**39.9%**	
Ovaries	1	4	Af	D	≥2y				(Lawrence, 1956)
	1	3	Both	D	≥2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	3	6	Both	D	≥2y				(Rankin, 1961b)
Sum. Ovaries	**5**	**18**				**27.8%**	**12.5%**	**50.9%**	
Prostate	1	1	Af	D	≥2y				(Larsen et al., 1981)
	3	6	Af	Both	≥2y				(Larsen & Kopecky, 1970)
Sum. Prostate	**4**	**7**				**57.1%**	**25.0%**	**84.2%**	
Seminal vesicles	2	3	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	6	6	Af	Both	≥2y				(Larsen & Kopecky, 1970)
	1	1	Af	D	≥2y				(Larsen et al., 1981)
Sum. Seminal vesicles	**9**	**20**				**45.0%**	**25.8%**	**65.8%**	
Testes	0	10	As	D	<2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	2	4	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	0	6	Af	Both	≥2y				(Larsen & Kopecky, 1970)
	0	1	Af	D	≥2y				(Larsen et al., 1981)
	1	1	Af	D	≥2y				(Lawrence, 1956)
Sum. Testes	**3**	**22**				**13.6%**	**4.7%**	**33.3%**	
Uterus	18	148	Both	D	≥2y				(Kopecky, Larsen & Merkal, 1967)
	1	3	Both	D	≥2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	5	7	Both	D	≥2y				(Rankin, 1961b)
Sum. Uterus	**24**	**163**				**14.7%**	**10.1%**	**21.0%**	
Bladder	0	7	Both	D	≥2y				(Rankin, 1961b)
	1	5	As	D	≥2y				(Rankin, 1961a)
Sum. Bladder	**1**	**12**				**8.3%**	**1.5%**	**35.4%**	

Annex 1.15

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Fetus	9	24	NA	NA	NA				(Doyle, 1958)
	2	2	Af	D	≥2y				(Koenig et al., 1993)
	5	24	Af	D	NA				(Lawrence, 1956)
	9	407	Both	D	≥2y				(Seitz et al., 1989)
	5	58	As	D	≥2y				(Sweeney et al., 1992b)
Sum. Fetus	**30**	**515**				**5.8%**	**4.1%**	**8.2%**	
Mammary Gland	4	4	Af	D	≥2y				(Alexejeff-Goloff, 1929)
	2	34	Af	D	≥2y				(Doyle, 1954)
	1	1	Both	D	≥2y				(Rankin, 1958)
	0	2	Both	D	<2y				(Rankin, 1958)
	0	5	As	D	≥2y				(Rankin, 1961a)
	2	6	Both	D	≥2y				(Rankin, 1961b)
	2	4	Af	D	≥2y				(Taylor et al., 1981)
Sum. Mammary Gland	**11**	**56**				**19.6%**	**11.3%**	**31.8%**	
Diaphragm muscle	2	5	Both	B	≥2y				(Alonso-Hearn et al., 2009)
	4	42	Both	D	≥2y				(Alonso-Hearn et al., 2009)
Muscle extensor carpi radialis	0	40	Af	D	≥2y				(Antognoli et al., 2008)
Heart muscle	2	40	Af	D	≥2y				(Antognoli et al., 2008)
Muscle longissimus colli	0	40	Af	D	≥2y				(Antognoli et al., 2008)
Sum. Muscle	**8**	**167**				**4.8%**	**2.4%**	**9.2%**	
Pharynx	4	21	As	D	<2y				(Payne & Rankin, 1961b)
	0	8	As	D	<2y				(Payne & Rankin, 1961a)
	0	8	As	D	≥2y				(Payne & Rankin, 1961a)
Sum. Pharynx	**4**	**37**				**10.8%**	**4.3%**	**24.7%**	

Annex 1.16

Tissue sampled	Positives	Samples collected	Clinical Status	Husb. Type	Age range	Prev.	95% CI		Reference
Sup.Mam. LN and Mam.gl.	8	16	Both	Nd	Nd				(Brady et al., 2008)
Abdominal organs	8	18	Both	Nd	Nd				(Brady et al., 2008)
Thoracic organs	14	21	Both	Nd	Nd				(Brady et al., 2008)
Plasma	1	7	Af	D	≥2y	14.3%	2.6%	51.3%	(Koenig et al., 1993)
Colostrum	10	126	As	D	Nd	7.9%	4.4%	14.0%	(Streeter et al., 1995)
Semen	1	1	As	D	≥2y				(Ayele, Bartos, Svastova & Pavlik, 2004)
	1	1	Af	Both	≥2y				(Larsen & Kopecky, 1970)
	1	1	Af	D	≥2y				(Larsen et al., 1981)
Sum. Semen	**3**	**3**				**100.0%**	**43.8%**	**100.0%**	
Milk	3	4	Af	D	≥2y				(Alexejeff-Goloff, 1929)
	15	86	As	D	Nd				(Ayele et al., 2005)
	5	11	Af	D	≥2y				(Giese & Ahrens, 2000)
	43	1493	As	D	Nd				(Jayarao et al., 2004)
	2	24	As	D	Nd				(Paolicchi et al., 2003)
	9	211	As	D	Nd				(Pillai & Jayarao, 2002)
	1	20	Af	D	≥2y				(Smith, 1960)
	0	13	As	D	Nd				(Smith, 1960)
	3	126	As	D	Nd				(Streeter et al., 1995)
	9	77	As	D	≥2y				(Sweeney et al., 1992b)
	9	26	Af	D	≥2y				(Taylor et al., 1981)
Sum. Milk	**99**	**2091**				**4.7%**	**3.9%**	**5.7%**	
Collection chamber flush fluid	2	6	Af	D	≥2y	33.3%	9.7%	70.0%	(Koenig et al., 1993)
Uterine flush fluid	3	4	Af	D	≥2y	75.0%	30.1%	95.4%	(Rohde & Shulaw, 1990)
Mammary Gland flush fluid	0	7	Af	D	≥2y	0.0%	0.0%	35.4%	(Koenig et al., 1993)
Sum. All tissues analyzed	**2297**	**22485**				**10.2%**	**9.8%**	**10.6%**	