A Diagnostic and Control Programme for Johne’s Disease on Irish Dairy Farms

Aideen Kennedy
Department of Biological Sciences. Cork Institute of Technology, Cork, Ireland.

Follow this and additional works at: https://sword.cit.ie/allthe

Part of the Bacteriology Commons

Recommended Citation

This Doctoral Thesis is brought to you for free and open access by the Dissertations and Theses at SWORD - South West Open Research Deposit. It has been accepted for inclusion in Theses by an authorized administrator of SWORD - South West Open Research Deposit. For more information, please contact sword@cit.ie.
A diagnostic and control programme for Johne's disease on Irish dairy farms

Aideen Kennedy

PhD 2017
A diagnostic and control programme for Johne’s disease on Irish dairy farms

A Thesis Presented for the Degree of Doctor of Philosophy

by

Aideen Kennedy M.V.B.

Department of Biological Sciences

1Dept. of Biological Sciences, Cork Institute of Technology, Bishopstown, Co, Cork

2Animal and Grassland research and Innovation Centre, Teagasc, Moorepark, Fermoy, Co. Cork

Supervisors

1Dr Jim O’Mahony
2Dr Riona Sayers

Submitted to Cork Institute of Technology, May, 2017
Declaration

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree of Doctor of Philosophy, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Student signature: ______________________

Date: ______________________

[12/19/2017]

[18/10/17]
Acknowledgements

I would like to acknowledge and thank those who helped make completion of my thesis possible.

Dr. Riona Sayers; for providing invaluable guidance, support and help with all aspects of the project and for enabling my transition from practice to research! The assistance with the many mystifications presented along the way was invaluable, as was the general help and advice. Tá mé fior-bhuioch duit!

Dr. Jim O’Mahony; for endless encouragement and positivity towards the work. Thanks for all the help and advice.

Noel Byrne; for an immense contribution, numerous early morning starts, general advice and overall help without which the project would not have been possible

Dr. Pat Dillon, Head of Centre, for the facilities and resources made available, and the Teagasc Walsh Fellowship Scheme for funding my research.

All the Teagasc farm staff who helped with the trial work, especially all those in Curtins and Moorepark for their help and patience with collection of samples. Also those in Cork RVL, DAFM and UCD who assisted with the project.

To my housemates, office mates and the many friends I have made along the way, thanks for the friendship, support, assistance with various thesis related issues, and mutual tension-relieving grumbling sessions!

To my siblings Emer and Philip; Philip for witty perspective, Emer for providing invaluable advice, listening to my endless musings and providing guidance in all things PhD related!

Most importantly to my parents, for their immense support and encouragement in everything.
Thesis Abstract

Title: A diagnostic and control programme for Johne's disease on Irish dairy farms.

Candidate: Aideen Kennedy

Johne's disease (JD) is an enteritis of ruminants with potential zoonotic implications. JD diagnosis and control present difficulties given JDs prolonged subclinical nature. In order to improve JD control in Ireland, PhD aims included conducting a national survey documenting high-risk husbandry practices employed on Irish farms, thereby targeting areas for improved management. Furthermore as JD has been associated with on-farm economic losses internationally, this research programme aimed to analyse production losses associated with testing JD ELISA positive on Irish dairy farms. A longitudinal study was also conducted to assess if successful JD control can be achieved using gold-standard protocols. Due to the prolonged nature of the disease, diagnosis of MAP is notoriously difficult. None of the available tests for MAP report perfect test sensitivity or specificity. A further complicating issue in Ireland is the high level of bovine tuberculosis (bTB) testing that is conducted. Multiple investigations relating to bTB-testing and its relationship with MAP diagnostics were conducted. Across all investigations, 312 farms were recruited, samples were collected from 4500 cows, and 10000 test results were generated (ELISA, PCR, faecal culture). Irish farms engaged in high-risk management practices facilitating JD transmission, however, no significant associations were identified between JD ELISA positivity and production parameters. On-farm sero-prevalence was decreased by implementation of gold standard protocols. Eradication was not achieved, however, highlighting the long-term commitment required for effective JD control. Annual bTB testing was associated with an increased prevalence of JD ELISA positive results and indicates that sampling for the purposes of JD surveillance should be avoided for 71 days post-TB test administration. Antibody responses and interferon-γ production were significantly increased post-bTB testing. As cell-mediated immunity is particularly important in the control of JD systemically, bTB testing may be contributing to the low levels of clinical JD in Ireland and warrants further investigation. This research programme has greatly contributed to our knowledge of JD in Ireland and improves understanding of practical measures necessary to interpret ELISA diagnostics and aid JD control.
Abbreviations

AHI  Animal Health Ireland
AP   Apparent Prevalence
bTB  Bovine Tuberculosis
CA   Calving area
CST  Comparative Skin Test
DTH  Delayed Type Hypersensitivity
EBI  Economic Breeding Index
ELISA Enzyme Linked Immunosorbent Assay
FRx  Friesian/Friesian crosses
GEE  Generalised estimating equations
HACCP Hazard analysis and Critical Control point
ICBF Irish Cattle Breeding Federation
IFN-γ Interferon gamma
IgG  Immunoglobulin G
JD   Johne's Disease
M. bovis Mycobacterium bovis
MAA  Mycobacterium avium
MAP  Mycobacterium avium subspecies paratuberculosis
MF   Milk fat
MP   Milk protein
MY   Milk yield
OR   Odds ratio
PCR  Polymerase Chain Reaction
PPD  Purified protein derivative
SCC  Somatic Cell Count
Se   Sensitivity
SICCT Single intradermal cervical comparative test
SIT  Single intradermal test
Sp   Specificity
TP   True Prevalence
VRAMP Veterinary risk assessment and management planning
JEX  Jersey/Jersey crosses
NRX  Norwegian Red/Norwegian Red crosses
Table of Contents

TABLE OF CONTENTS......................................................................................6

CHAPTER 1: INTRODUCTION.........................................................................12

CHAPTER 2: LITERATURE REVIEW...............................................................17

2.1 INTRODUCTION..........................................................................................18
2.2 MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS (MAP) .........19
  2.2.1 Clinical Signs........................................................................................19
  2.2.2 Susceptible Species..............................................................................19
  2.2.3 Transmission of MAP...........................................................................20
  2.2.4 Risk Factors..........................................................................................22
2.3 IMMUNOLOGY.............................................................................................25
  2.3.1 Overview of the immune system.........................................................25
  2.3.2 MAP ENTRY..........................................................................................27
    2.3.3 Immune Response induced by MAP..................................................28
    2.3.4 MAP Survival....................................................................................29
2.4 PATHOLOGY................................................................................................30
  2.4.1 Gross Pathology...................................................................................30
  2.4.2 Histopathology....................................................................................32
2.5 DIAGNOSTICS...........................................................................................33
  2.5.1 Introduction..........................................................................................33
  2.5.2 ELISA..................................................................................................35
  2.5.3 Culture..................................................................................................36
  2.5.4 Polymerase Chain Reaction.................................................................37
  2.5.5 Interferon gamma................................................................................38
  2.5.6 Intradermal testing..............................................................................38
  2.5.7 Necropsy.............................................................................................39
  2.5.8 Ziel- Nieslon.......................................................................................39
2.6 PREVALENCE............................................................................................40
  2.6.1 Prevalence Overview...........................................................................40
2.7 ECONOMICS...............................................................................................41
  2.7.1 Economic Losses..................................................................................41
2.8 CONTROL PROGRAMME..........................................................................45
  2.8.1 Introduction..........................................................................................45
  2.8.2 Management Practices.......................................................................46
  2.8.3 Challenges...........................................................................................47
2.9. TUBERCULOSIS.......................................................................................48
  2.9.1 Overview............................................................................................48
  2.9.2 Irelands TB eradication Programme...................................................49
  2.9.3 Interferon Gamma Test........................................................................52
2.10. METHODS...............................................................................................52
  2.10.1 Statistics............................................................................................52
  2.10.2 Epidemiology.....................................................................................56
  2.10.3. Statistics in Epidemiology.................................................................56
  2.10.4 PCR....................................................................................................58
  2.10.5 Faecal Culture....................................................................................60
  2.10.6 ELISA Technique.............................................................................62
CHAPTER 3: A SURVEY OF MANAGEMENT PRACTICES ON IRISH DAIRY FARMS WITH EMPHASIS ON RISK FACTORS FOR JOHNE'S DISEASE TRANSMISSION ........................................................................................................65

3.1 ABSTRACT ........................................................................................................66
3.2 INTRODUCTION ................................................................................................67
3.3 MATERIALS AND METHODS ...........................................................................69
  3.3.1 Survey Procedure ..............................................................................69
  3.3.2 Survey Questionnaire ........................................................................70
  3.3.3 Descriptive Analysis and Herd Classification ....................................74
  3.3.4 Statistical Analysis ............................................................................74
3.4 RESULTS ...........................................................................................................75
  3.4.1 JD Questionnaire Descriptive and Statistical Analysis .......................75
  3.4.2 New-born Calf Management ................................................................76
  3.4.3 Hygiene and Faeces Management .....................................................79
  3.4.4 Biosecurity Questionnaire Descriptive Analysis ...............................79
3.5 DISCUSSION ...................................................................................................82
3.6 CONCLUSIONS ..............................................................................................86

CHAPTER 4: ANALYSIS OF JOHNE'S DISEASE ELISA STATUS AND ASSOCIATED PERFORMANCE PARAMETERS IN IRISH DAIRY COWS ........................................................................................................87

4.1 ABSTRACT ........................................................................................................88
4.2 INTRODUCTION ................................................................................................89
4.3 MATERIALS AND METHODS .........................................................................92
  4.3.1 Study Population and Sampling .........................................................92
  4.3.2 Sample Testing ..................................................................................93
  4.3.3 Individual Cow Performance Data ...................................................93
  4.3.4 Survey Data .......................................................................................94
  4.3.5 Dataset Construction .........................................................................94
  4.3.6 Data Analysis .....................................................................................94
  4.3.7 Prevalence Calculation .......................................................................95
  4.3.8 Associations between cow performance and MAP ELISA status ....96
  4.3.9 Associations between breed, herd size, parity, EBI and MAP ELISA status ........................................................................................................97
  4.3.10 Associations between farm management practices and herd MAP status ........................................................................................................97
4.4 RESULTS ..........................................................................................................97
  4.4.1 Descriptive Data ................................................................................97
  4.4.2 Prevalence .........................................................................................102
  4.4.3 Milk ELISA Results ..........................................................................102
  4.4.4 Management Practices Survey ........................................................104
  4.4.5 Production Data ...............................................................................106
  4.4.6 Mixed model analysis of production parameters ..............................106
  4.4.7 Associations between breed, herd size, parity, EBI and MAP ELISA status ........................................................................................................107
  4.4.8 Associations between farm management practices and herd MAP status ........................................................................................................107
4.5 DISCUSSION .....................................................................................................109
4.6 CONCLUSION ..................................................................................................113
CHAPTER 5: RESULTS OF A FIVE YEAR JOHNE'S DISEASE CONTROL PROGRAMME ............................................................................................................... 114

5.1 ABSTRACT .................................................................................................................. 115
5.2 INTRODUCTION .......................................................................................................... 116
5.3 MATERIALS AND METHODS .................................................................................... 118
  5.3.1 Study Herd ........................................................................................................... 118
  5.3.2 Sample Protocol ................................................................................................ 120
  5.3.3 Sample preparation and analysis ....................................................................... 121
  5.3.4 Management Protocols ...................................................................................... 123
  5.3.5 Selection criteria for clinical and post-mortem examination (PM) .................. 124
  5.3.6 Data and Statistical Analysis ............................................................................ 125
  5.3.7 Testing costs ...................................................................................................... 126
5.4 RESULTS .................................................................................................................... 126
  5.4.1 Herd clinical history ......................................................................................... 126
  5.4.2 Year-by-year herd results ................................................................................. 126
  5.4.3 Statistical Analysis ............................................................................................ 137
  5.4.4 Cost of programme .......................................................................................... 137
5.5 DISCUSSION .............................................................................................................. 138
5.6 CONCLUSION ............................................................................................................ 143

CHAPTER 6: THE SINGLE INTRADERMAL CERVICAL COMPARATIVE TEST INTERFERES WITH JOHNE'S DISEASE ELISA DIAGNOSTICS ...... 144

6.1 ABSTRACT .................................................................................................................. 145
6.2 INTRODUCTION .......................................................................................................... 146
6.3 MATERIALS AND METHODS .................................................................................... 147
  6.3.1 Study Herd ........................................................................................................ 147
  6.3.2 Sample Collection ............................................................................................. 148
  6.3.3 Data Analysis .................................................................................................... 150
6.4 RESULTS .................................................................................................................... 151
  6.4.1 TB Test ............................................................................................................. 151
  6.4.2 MAP ELISA ....................................................................................................... 151
  6.4.3 Faecal Samples .................................................................................................. 157
6.5 DISCUSSION .............................................................................................................. 157
6.6 CONCLUSIONS .......................................................................................................... 162

CHAPTER 7: INVESTIGATIONS AND IMPLICATIONS OF ASSOCIATIONS BETWEEN MYCOBACTERIAL PURIFIED PROTEIN DERIVATIVE HYPERSENSITIVITY AND MAP-ANTIBODY ELISA IN IRISH DAIRY COWS. .................................................................................................................................................. 163

7.1 ABSTRACT .................................................................................................................. 164
7.2 INTRODUCTION .......................................................................................................... 165
7.3 MATERIALS AND METHODS .................................................................................... 166
  7.3.1 Study Herd ........................................................................................................ 166
  7.3.2 Comparative Skin Test ....................................................................................... 166
  7.3.3 Blood Sampling ................................................................................................ 166
  7.3.4 Statistical Analysis ............................................................................................ 167
  7.3.5 Faecal Sampling 2016 ....................................................................................... 167
7.4 RESULTS .................................................................................................................... 167
7.5 DISCUSSION .............................................................................................................. 171
7.5 CONCLUSIONS .......................................................................................................... 172
List of Figures

2.1 Transmission cycle of MAP ............................ 21
2.2 Immune Response ...................................... 26
2.3 Thickened intestines with dilated serosal lymphatics .. 31
2.4 Mucosal section showing thickened corrugated intestines .. 31
2.5 Histopathology section from a cow infected with JD .... 33
2.6 Stained smear showing acid fast bacilli .................. 40
2.7 Measurement of skin thickness .......................... 50
2.8 Bell-shaped curve of a normal distribution .............. 53
2.9 Diagram of a box plot ................................... 55
2.10 Antibody binding site ................................. 63
3.1 Responses to Biosecurity Questionnaire ....... 81
4.1 Map showing location of study farms .................... 92
4.2 Proportion of animals tested belonging to each breed. 98
4.3 Proportion of animals tested belonging to each parity ... 99
4.4 Box plot showing range of S/P ratios across all herds ... 101
4.5 Proportion of animals testing positive per parity ........ 103
4.6 Proportion of positive results recorded across each breed 103
4.7 Scatter Plot ............................................ 104
4.8 Responses to survey questions .......................... 105
5.1 Location of the study herd shown in red ................ 119
5.2 Herd prevalence of serum ELISA positives ............ 128
5.3 Trends in serum S/P% ratio ............................ 129
5.4 Trends in serum S/P ratios of positive cows ............ 132
5.5 Classical signs of JD .................................... 134
5.6 Percentage of the herd positive on JD ELISAs .......... 155
6.1 Box plot serum ......................................... 156
6.2 Box plot milk ........................................... 156
6.3 Box plot milk ........................................... 156
6.4 Variation in period of influence of SICCT ................ 158
7.1 Box plots showing the differences in MAP S/P ratios ... 170
8.1 Measurement of oedematous lesion .................... 177
8.2 Box plots showing IFN-Y and MAP ELISA response .... 187
8.3 Scatter plot ............................................ 188
List of Tables

3.1 Responses to the Johne’s disease questionnaire 72
3.2 Significant associations between variables 76
3.3 Spearman correlation values between dependent variables 78
4.1 Estimates for use in true prevalence calculation 96
4.2 Breakdown of positives across parity and breed. 100
4.3 Results from multilevel mixed model analysis 106
4.4 Significant associations MAP ELISA & independent variables 108
5.1 Sampling regime across each year of the study 121
5.2 History of cows with a minimum of one ELISA positive 131
5.3 Highest and lowest serum ELISA S/P results of PM cows 135
5.4 Univariable logistic regression 137
5.5 Associations between PM signs and independent variables 138
6.1 Timetable of serum and milk samples and dates of SICCT 149
6.2 Multivariable GEE analysis of milk ELISA 153
6.3 Multivariable GEE analysis of serum ELISA 155
7.1 Results from GEE analysis 169
8.1 Univariable analysis 185
8.2 Associations between IFN-y and independent variables 186
8.3 Associations between ELISA response and independent variables 186
Chapter 1: Introduction
Initiatives such as Origin Green (www.origingreen.ie), which promote a wholesome and sustainable image of Irish agriculture, allow Irish dairy farmers command a premium price for their produce. Key to maintaining Ireland's "green" image is optimum animal health and welfare. This not only contributes to our branded image, it is also an important factor in achieving on farm efficiency, as healthy animals are more efficient compared to diseased livestock (Dehove et al., 2012). Sustainable, efficient food production therefore will be dependent on disease control. Furthermore, due to European milk quota abolition, Irish dairy farmers are producing milk in an unsupported and unrestricted global market, therefore dairy production systems will have to operate at optimal efficiency in order to withstand global milk price fluctuations (Sayers, 2014). Given the value of the agri-food sector to the Irish economy (7.6% of Ireland's economy-wide gross value added (GVA) (measure of the value of goods and services produced in an area), (DAFM, 2014)), it is imperative we maximize our efficiency and productivity while also promoting optimal animal health and welfare practices.

A recent Delphi study by Animal Health Ireland (AHI) was conducted to illicit the opinions of industry experts on high-priority non-regulatory disease/conditions relating to their cost, the impact their occurrence has on international perception and the impediment they cause to international market access. As part of this study Johne's disease (JD) was prioritised as one of the most important animal health issues facing the Irish livestock industry (More et al., 2010). Johne's disease, a chronic incurable enteritis of ruminants, is caused by infection with Mycobacterium avium subspecies paratuberculosis (MAP). Animals are normally infected as calves but due to the slowly progressive nature of the disease, clinical signs of weight loss, diarrhoea, emaciation and eventual death, usually do not occur until adulthood (Sweeney et al., 2012). Control programmes for JD have been initiated to address animal health and welfare concerns and tackle the reported economic losses associated with JD (Garcia and Shalloo, 2015). A further area of concern relating to JD is the speculated zoonotic link between MAP in milk causing Crohn's disease in humans (Hermon-Taylor, 2009). Should this hypothesised link ever be proven it
is estimated to cost the global dairy industry billions of dollars (Groenendaal and Galligan, 2003). Ireland supplies 15% of all global infant formulae (International Dairy Federation, 2013), as such in order to maintain market access it is imperative we minimise levels of MAP in our milk.

A large number of high risk management practices that increase the likelihood of JD infection have been identified, such as pooling colostrum and allowing calves access to the faeces of adult cows (Doré et al., 2012). Little research has been conducted however into the level of implementation of such high risk JD related management practices on Irish dairy farms. As part of this body of work, a survey was conducted on a population of farmers geographically representative of the national farm population, to identify if Irish farmers were engaging in low or high risk management practices potentially contributing to disease transmission. Results from this study provide key baseline data of management practices implemented on Irish dairy farms, and will aid Animal Health Ireland’s (AHI) newly announced JD control programme which commences in September 2017.

Conflicting reports exist in the literature relating to the economic impact JD has on Irish dairy farms. While Barrett et al. (2006) reported losses, Hoogendam et al. (2009) reported no significant effect of ELISA status on dairy cow production parameters. Due to quota abolition in 2015, herd size in Ireland is increasing (Shalloo et al., 2012). Large herd size is a known risk factor for testing JD positive (Scott et al., 2006). This study, therefore, aimed to investigate production losses associated with testing JD ELISA positive using herds larger than the national average. Secondary objectives included investigation of risk factors associated with testing MAP ELISA positive and will identify specific practices that will need to be tackled on farm in order to minimise disease transmission.
Internationally, longitudinal studies have been conducted to monitor the success of JD control programmes (Ferrouillet et al., 2009, Collins et al., 2010). No previous longitudinal study has been conducted on an Irish dairy farm to investigate if JD sero-prevalence could be reduced following control programme implementation. To address this knowledge gap a "best practice" management plan (Sweeney et al., 2012) was implemented on an Irish dairy farm using serial ELISA testing as the basis for initial identification of MAP positive animals. Best practice dry-cow management practices, calf management practices and culling of high-risk cows also formed part of the control programme.

A number of current control programmes for JD involve breaking the cycle of disease transmission through identification and removal of infected animals (Nielsen et al., 2007, Ferrouillet et al., 2009, Collins et al., 2010). Diagnosis of JD however is notoriously difficult. None of the available tests for MAP report perfect test sensitivity or specificity (Nielsen and Toft, 2008). A further complicating issue in Ireland is the high level of bovine tuberculosis (bTB) testing that is conducted (Good et al., 2007). No previous study has been conducted investigating the impact of bTB testing on blood and milk MAP ELISA diagnostics in a MAP seropositive herd. This study aimed to address this knowledge gap, and provide definitive guidelines on the appropriate timing of sample collection for MAP diagnostics in the period post bTB test administration.

As the bTB test is mandatory in Ireland (Good, 2006), and there is a close genetic relationship between MAP and Mycobacterium bovis (Thorel et al., 1990), we also investigate whether the mandatory bTB test could be incorporated into diagnostic strategies for JD. Results from this study will aim to establish if combining both the response to the bTB test and post bTB MAP ELISA response can potentially aid diagnosis of subclinical MAP infection. Finally, a novel study was also conducted, investigating whether the administration of the bTB skin test leads to a systemic increase in production of the cytokine interferon gamma, a cytokine known to be important in the control
of JD (Koets et al., 2015). Results from this study will potentially determine if the bTB test is contributing to JD control in Ireland.

To summarise this thesis sought to

1. Provide baseline data relating to management practices and risk factors placing Irish farmers at increased risk of disease transmission.
2. Assess associations between testing MAP ELISA positive and dairy cow production parameters.
3. Implement an Irish appropriate testing and control programme to reduce within herd MAP prevalence.
4. Examine the impact of bTB tests on MAP ELISA tests, while also attempting to incorporate bTB testing as part of MAP diagnostic strategies.
5. Identify if bTB testing potentially aids MAP control.

This study seeks to provide guidelines to dairy farmers and vets to allow them to implement appropriately timed diagnostic regimes and incorporate effective control programmes for JD. Ultimately it is hoped that guidelines resulting from this thesis will aid MAP diagnosis, reduce the transmission of JD, minimise MAP levels in Ireland thereby improving animal welfare and contributing to the competitiveness and efficiency of the Irish dairy industry.
Chapter 2: Literature Review
2.1 Introduction

Mycobacteria, belonging to the genus *Mycobacterium*, the only genus in the Mycobacteriaceae family, are a leading cause of infection in domesticated animals and wildlife (Rastogi et al., 2001). The mycobacterial species, *Mycobacterium avium*, is divided into a number of subspecies including *M. avium* subsp. *avium* (MAA), *M. avium* subsp. *silvaticum* and *M. avium* subsp. *hominissuis* (Thorel et al., 1990, Turenne et al., 2008). A further subspecies is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the etiological agent of Johne’s disease, an acid fast, gram positive organism, containing a complex lipid rich cell wall (Harris and Barletta, 2001). MAP was initially identified in 1895 by H.A. Johne and L. Frothingham. In 1910, F.W. Trowt fulfilled Koch’s postulates by growing MAP and reproducing the disease in an experimentally infected animal and named it *M. enteritis chronicae pseudotuberulosae bovis john* (Behr and Collins, 2010). Since 1923 the disease has been known as Johne’s disease (JD) or paratuberculosis (Berney et al., 1923).

MAP is the slowest growing of the cultivable mycobacteria (Lambrecht et al., 1988). Dependence on mycobactin for growth allows phenotypical differentiation of MAP from MAA (Thorel et al., 1990). Analysis of the MAP genome has allowed identification of an insertion sequence IS 900 (Collins et al., 1989), which genotypically distinguishes MAP from MAA; however the close genetic similarity is reflected in a large number of common antigens (Thorel et al., 1990). Distinct strains of MAP exist including a sheep type (Type S) and a cattle strain (Type C) (Collins et al., 1990). Differences exist between both the cattle and sheep strains. Verna et al., (2007) inoculated lambs with sheep strains and additional lambs with cattle MAP strains. The pathology of lambs infected with the bovine strain were characterized by focal lesions mainly in the mesenteric lymph nodes, the presence of fibrous tissue, and, occasionally, necrosis in the granulomas as well as the presence of numerous giant cells. Lesions in lambs inoculated with sheep strains were more severe and occurred mainly in the intestinal lymphoid tissue; necrosis, fibrosis or giant cells were never detected in this group. As the correlation between strain and host
species is not absolute it was proposed that strain types be referred to as Type I (Type S) or Type II (Type C) (Stevenson et al., 2002) although both designations are still in use.

2.2 *Mycobacterium avium* subspecies *paratuberculosis* (MAP)

2.2.1 Clinical Signs

Clinical signs of Johne's disease include weight loss despite a normal appetite, diarrhoea, submandibular oedema, emaciation, lethargy, and eventual death as currently there is no effective treatment for JD (Sweeney et al., 2012). Latency is a common feature of mycobacterial diseases (Nielsen et al., 2013). Animals can remain sub-clinical without showing any external signs of the disease for many years. Clinical disease most frequently occurs in cattle aged 2 – 5 years (Doyle, 1956). Three target conditions are described, including infected, infectious and affected (Nielsen and Toft, 2008).

Affected animals have lost control of the disease and display clinical signs of weight loss and diarrhoea. Evidence of the disease can be documented at post mortem, histopathology or by culturing the bacterium in faeces (Nielsen and Toft, 2008).

Infectious animals are defined as those shedding MAP at the time of testing. They include “affected animals”, but may also include non-infected animals that have recently ingested the organism and are passively shedding it.

Infected animals carry MAP intracellularly. The case definition involves entry and persistence of MAP for a period long enough to promote an immune response. There is no specific cut-off point as to when the immune response event occurs.

2.2.2 Susceptible Species

JD predominantly occurs in cattle and sheep in temperate climates with adequate rainfall (Ayele et al., 2001). A number of claw hoofed species are
susceptible to MAP. Goats are readily infected (Stewart et al., 2006) and a number of species of deer have also been identified as hosts of MAP, including farmed red deer in Ireland (Power et al., 1993).

Wild animals are often considered reservoirs for infectious disease (Simpson, 2002). The badger has long been associated with the failure to eradicate bovine tuberculosis (bTB) in Ireland (Corner, 2006), and birds are commonly implicated as wildlife reservoirs of salmonella and other pathogenic microorganisms (Hubálek, 2004). MAP has been isolated from a number of wildlife species including rabbits, foxes, crows, rooks, jackdaws, rats, wood mice, hares, badgers and stoats (Beard et al., 2001). Paratuberculosis is a notoriously difficult disease to control; the participation of wildlife in the spread of the disease may partially account for this difficulty (Daniels et al., 2003).

2.2.3 Transmission of MAP

Transmission of MAP, much like other faecal-oral transmitted pathogens e.g. salmonella, is facilitated by direct ingestion of contaminated faeces, or via the ingestion of foodstuffs contaminated with MAP infected faeces (Behr and Collins, 2010). JD infected cows may also shed MAP in colostrum and milk (Streeter et al., 1995, Sweeney, 1996). In utero infection (Seitz et al., 1989, Sweeney et al., 1992a), is estimated to occur in 39% of clinically infected dams and 9% in subclinical animals (Whittington and Windsor, 2009). Indirect transmission can also occur with environmental contamination contributing to disease persistence in high prevalence herds.
Figure 2.1. Transmission cycle of MAP.

Infection can occur as a calf via ingestion of MAP contaminated faeces, colostrum, milk or in utero. Even though infected, the animal can appear healthy for many years. The disease is slowly progressive with clinical signs of weight loss and diarrhoea.
2.2.4 Risk Factors

Importation of animals from abroad has been reported as a key risk factor in the establishment of JD in Ireland (Barrett et al., 2011). The introduction of the single European market in 1992 allowed free movement of animals within the European Union. Prior to this, less than 100 cases of JD were identified in Ireland. Between 1992 and 2004, opening of market access saw the influx of approximately 85,000 animals into the country. Simultaneously, between 1995 and 2002, the number of reported clinical JD cases in Ireland increased to 232, the increased levels of JD corresponding with livestock importation (Barrett et al., 2006).

Large herd sizes are associated with an increased risk of MAP infection (Wells, 2000, Scott et al., 2006). In most commercial herds the initial introduction of MAP usually occurs as the result of the purchase of an infected but clinically normal animal (Sweeney, 1996). Once MAP is introduced to a herd, infection with MAP is primarily understood to occur as a calf (Taylor, 1953). Animals less than six months are believed to be the most susceptible (Windsor and Whittington, 2010). Neonates are considered to be the highest risk of acquiring MAP infection due to increased permeability of intestines during the first 24 h of life and an immature immune system in young calves (Chase et al., 2008). The younger that animals are exposed to MAP, the more likely they are to start shedding early and progress to clinical disease (Windsor and Whittington, 2010). Older animals appear less susceptible, however infection of adult animals may still occur (Doyle, 1956, Windsor and Whittington, 2010, Mortier et al., 2013).

The major route of MAP transmission is faecal–oral (Lombard, 2011). The contact of calves with faeces from adult cows is the most important risk factor precipitating the spread of MAP (Doré et al., 2012). Primary environmental risk factors for infection are faecal contamination of the calving pen or the udder (Lombard, 2011). Management of the peri-parturient cow and the calving area are therefore of critical importance.
Factors associated with increased risk of JD transmission relating to calving area management include use of group housing for peri-parturient cows (Wells, 2000). Tiwari et al. (2009), also reported an increased risk of infection associated with the presence of more than one cow in the maternity pen. Levels of risk associated with indoor versus outdoor calving have likewise been examined, with Ridge et al. (2005) finding an increased risk of MAP infection when calving occurred indoors versus a paddock. The increased availability of space and the lower levels of faecal contamination were cited as advantages to calving outdoors (Radia et al., 2013), although that study does acknowledge suitable weather conditions need to be available. A protective effect of using individual calving pens while the cows are at grass has been demonstrated (Çetinkaya et al., 1997). Similarly Pithua et al. (2013) also found that while indoors, individual calving pens versus group pens were effective in delaying the exposure of calves to MAP. As the contact of calves with faeces is perceived as the greatest transmission risk, maintaining adequate hygiene in the calving area is also of great importance (Benedictus et al., 2008). Cleaning of the maternity pen after each use (Johnson-Ifeearulundu and Kaneene, 1998) and adequate bedding (Nielsen, 2007) may be of value to decrease the risk of MAP transmission.

Cashman et al. (2008), showed that reduced attendance at calving led to an increase in MAP infection. Attendance at calving would potentially facilitate the prompt removal of calves, which is correlated with the apparent prevalence of MAP (Goodger et al., 1996). Prompt removal of calves also facilitates greater regulation of the new-born calf’s diet (Collins et al., 2010). Preventing sucking will negate the risk of faecal contamination from a dirty udder known to increase risk of MAP infection (Ansari-Lari et al., 2009), and the potential risk of MAP transmission via colostrum from a cow of unknown status. Collins et al. (2010) advise only feeding colostrum from test negative animals, as feeding colostrum from ELISA positive cows increased the risk of MAP infection (Dieguez et al., 2008). Nielsen et al. (2008) found that pooling of colostrum versus feeding
colostrum from the calf's own dam posed an increased risk of MAP infection. Multiple suckling (Cashman et al., 2008) or suckling of foster cows (Nielsen et al., 2008) has also been shown to increase the risk of MAP infection.

The use of waste milk poses a risk of transferring a number of bacteria (Selim and Cullor, 1997), not just MAP (Ridge et al., 2005). Muskens et al. (2003), found greater use of milk replacer in MAP sero-negative herds, while other studies have also advocated the use of milk replacer or pasteurised milk (Collins et al., 2010) however, it should be noted MAP has still been identified in pasteurised milk (Grant et al., 1996, Ellingson et al., 2005, Slana et al., 2008). Other management practices to be considered include avoiding housing calves with adults as this has been shown to increase risk of MAP infection (Dieguez et al., 2008). Equally, adequate separation of calf housing with adults and their manure is to be recommended (Goodger et al., 1996, Ridge et al., 2005), including avoiding sharing equipment between management groups.

While limiting faecal exposure in the calving area and post calving housing is helpful for reducing within herd prevalence of MAP (Radia et al., 2013), the survival techniques employed by MAP in the environment (Rowe and Grant, 2006) means that MAP can survive in grazing areas. Fecteau et al. (2010), showed that grazing yearling animals in pasture previously contaminated by MAP infected animals led to infection, showing that age resistance to infection can be overcome by pressure of infection. Manure spreading poses a risk as prolonged survival times of MAP has been reported in slurry (Jorgensen, 1977), necessitating careful farm management planning, to avoid spreading in fields where calves may potentially graze in the future.

Highlighting on farm management practices using veterinary risk assessment and management plans (VRAMP) is a commonly utilised tool in a number of control programmes (Geraghty et al., 2014), including Animal Health Ireland's (AHI) JD control programme. The VRAMP is a combined work between the farmer and a trained local vet familiar with the farm, to facilitate targeting of
specific high risk practices occurring on an individual farm. Repeat visits allow monitoring of successful implementation of management changes (see 2.8 Control Programme). Hazard analysis and critical control point (HACCP) plans also highlight risky practices in peri-parturient area management, calving, newborn calf management and colostrum management (McAloon et al., 2015). Highlighting risky practices and implementing improved management plans can help to reduce the prevalence of MAP on farm (Collins et al., 2010).

2.3 Immunology

2.3.1 Overview of the immune system

The immune system protects against microbial invasion and is essential for life (Tizard, 2013). Physical barriers such as the skin provide the first line of defence. The next line of defence involves non-specific or innate immune responses.

Innate Immunity:

Two types of signals trigger the innate immune defenses. One such signal is generated due the presence of invading microorganisms. The microorganisms are detected by sensing their characteristic surface molecules known as pathogen associated molecular patterns (PAMPs). Molecules released following the damage of tissues can also trigger innate responses. These are known as damage associated molecular patterns (DAMPs). PAMPs and DAMPs bind to pattern recognition receptors (PRRs). PRRs are found on many cells including macrophage and dendritic cells (Tizard et al., 2013). Toll-like receptors (TLRs) are a major group of PRRs. Stimulation of TLRs, following the binding of PAMPs leads to the activation of signaling pathways that result in the induction of cytokines. Additionally, stimulation of TLRs triggers dendritic cell maturation and results in the induction of costimulatory molecules and increased antigen-presenting capacity (Janeway et al., 2002). Therefore, not only does the innate immune response directly attack pathogens it is also helps to direct adaptive/specific immune responses.

Specific immunity:
This depends on specific recognition of invaders and the immune system launching an attack that is unique for that substance (Bonilla and Oettgen, 2010). It may take several days for the adaptive immune system to become effective. The adaptive immune system both recognises the microbes and retains a memory of the invasion, allowing a more efficient, faster response if a repeat invasion occurs.

Figure 1.2 Immune response. Primary and Memory Immune Response showing increased production of immunoglobulin G (IgG) following repeat exposure to the same antigen.

Lymphocytes are the key to the specific immune response (Tizard 2013). Activated B-cells differentiate into plasma cells, which secrete antibodies. T-cells attack the antigens directly. Cytotoxic T-cells (CD 8 cells) bind to antigens and directly destroy them. Helper T-cells (CD 4) secrete cytokines that help to activate B-cells, cytotoxic T-cells, NK cells and macrophage (Abbas et al., 1996). CD4 and CD8 refer to the presence of certain proteins in their plasma membranes. Two types of CD4 T-helper cell responses can be induced, the
Th1 and Th2 response (Mosmann et al., 1986). Interferon gamma release is characteristic of Th1 response, which activates the bactericidal properties of macrophage and induces B cells to make antibodies (Mosmann and Sad, 1996). All T-helper cells can be involved in stimulating antibody production. The immune response mediated by antibodies is referred to as humoral immunity. Cell mediated immunity relies on T-cells, including Th1, Th2 and cytotoxic T-cells.

2.3.2 MAP Entry

Following ingestion of MAP, the bacteria gets moved along the intestinal tract to the small intestine (Bannantine and Bermudez, 2013). The initial barrier against bacteria is the epithelium and its associated tight junctions (Bannantine and Bermudez, 2013). Microfold cells (M Cells) are specialised epithelial cells. M cells are located in the epithelium of the Peyers patches. They present antigens and microorganisms to the immune cells to induce an effective immune response (Kucharzik et al., 2000). A large number of transient ileal Peyers patches provide a route of MAP entry in young animals with an immature immune system (Koets et al., 2015). Ileal Peyers patches regress as the animal matures leaving the jejunal Peyers patches (Momotani et al., 1988).

Enterocytes also serve as a method of entry of MAP. MAP has an affinity for the surface of enterocytes and is readily internalised, with internalisation stimulating the production of chemokine’s (Pott et al., 2009). MAP gets translocated from the intestinal lumen to the submucosa, where ingestion by macrophages occurs (Lamont et al, 2012). Macrophages play a key role in host pathogen interaction. Not only are they chiefly involved in the destruction of MAP, but in the event of their subversion by MAP, macrophage transform into protective sanctuaries for the survival, proliferation and dissemination of MAP (Arsenault et al., 2014). In the first 24 hours of infection, changes develop rapidly in infected macrophage. Upon intracellular infection the macrophage attempts to attract T-cells through a pro-inflammatory response, while MAP tries to oppose this via the induction of anti-inflammatory pathways (Buza et al., 2003). The down regulation of a number of inflammatory proteins is associated with MAPs initial invasion.
et al., 2012). This may allow the bacteria to become established in the host (Bannantine and Bermudez, 2013). It also appears that when MAP is exposed to cow's milk there is enhanced invasion of the host (Bermudez et al., 2010).

2.3.3 Immune Response induced by MAP

It is generally believed that early subclinical MAP infections result in a cell mediated response and are associated with a delayed type IV hypersensitivity reaction and production of cytokines by T lymphocytes (Stabel, 2000).

A Th1 population of T-cells produce pro-inflammatory cytokines such as IFN-γ and a range of interleukins (IL 1a, II-6) (Coussens, 2001). Later in the course of the disease the Th1 response appears diminished. A Th2 response characterized by antibody production predominates (Stabel, 2000). This generally corresponds with disease progression. While a number of authors suggest there is a switch from a Th1 response to a Th2 controlled response (Stabel, 2000), other studies suggest the entire immune response is not working correctly and the cell mediated response is the first affected (Begg et al., 2011). In long term studies confirmation of a switch from a cell mediated to an antibody response has been difficult to confirm (Koets et al., 2015). A number of studies have shown both an IFN-γ and an antibody response without clear delineation of disease progression and clinical signs (Waters et al., 2003, Stewart et al., 2007).

Cytokine signalling is important in the host defences against mycobacteria (Coussens, 2004). IFN-γ has been established as an important cytokine in the fight against mycobacterial disease (Cooper et al., 1993, Flynn et al., 1993, Coussens, 2004). In response to MAP infection IFN-γ is one of the major cytokines activated (Burrells et al., 1999, Coussens, 2004) and would appear important in limiting infection. As MAP infection progresses newly recruited macrophage are drawn to the site of infection (Navarro et al., 1998). Newly recruited macrophages (if properly activated through interaction with IFN-γ) may be successful in the destruction of MAP (Zurbrick et al., 1988).

28
Dendritic cells and macrophages successful in degrading MAP travel to local lymph nodes. Here they present antigen and stimulate T-cells to produce IFN-γ. These T-cells are passed into the vascular system and journey to sites of MAP infection. At sites of infection, T-cells and macrophage produce IFN-γ possibly activating newly recruited macrophage.

2.3.4 MAP Survival

If macrophages are not successful at degrading MAP, there may be persistence and proliferation of MAP (Sigurðardóttir et al., 2004). Inside cells, MAP modulates the intracellular environment, survives and replicates, while also modulating cell surface expression of molecules and cytokine release (Arsenault et al., 2014).

Macrophages are the primary target cells for MAP in which to persist and replicate. MAP has extensive abilities to subvert the host innate immune system (Arsenault et al., 2014). One of the critical periods in the formation of a persistent infection occurs directly after MAP entry into the macrophage phagosome. Through programmed changes phagosomes undergo development to a late endosome (Arsenault et al., 2014). Fusion of the late endosomes with lysosomes, creates phagolysosomes. This is an environment that is suitable for the destruction of MAP (Arsenault et al., 2014). The survival of the internalized bacteria depends on its ability to disrupt the formation of the mature phagolysosome. In addition to preventing phagolysosome fusion, in order to survive and replicate Koets et al. (2015), report that MAP avoids being detected by manipulating signaling pathways of cytokines. In this way MAP induces an immunosuppressive environment between the intestinal lesion and the systemic immune response. Intracellular infection of intestinal dendritic cells leads to improper dendritic cell maturation (Koets et al., 2015). Dendritic cells that are not properly activated will not efficiently migrate to the local lymph node and will not function effectively as an antigen presenting cell. This will impede optimal induction of pro-inflammatory T-cells, and also impair migration of these T cells (Koets et al., 2015).
For those T-cells that do manage to migrate to lesions, recognition of infected macrophage is dependent on interaction of the T-cell receptor with macrophage expressed major histocompatibility complex (MHC) containing MAP specific molecules (Koets et al., 2015). Studies have shown however, MAP eludes detection by down regulation of MHC and interferes with expression of genes associated with antigen presentation (Purdie et al., 2012). MAP also interferes with IFN-γ induced signaling. Given the importance of IFN-γ, inhibition of this pathway is considered an important virulence mechanism (Arsenault et al, 2014)

In the latter stages of infection, infected macrophages are found throughout the ileum. The ileum is heavily infected and thickened preventing absorption of nutrients (Coussens, 2004). Protein losing enteropathy characterized by diarrhea, cachexia and death ensues. Only a small proportion of infected cows progress to clinical JD (Sweeney et al., 2012, Koets et al., 2015). Possible cited reasons including genetic resistance of the animal (Van Hulzen et al., 2014) and strain of MAP involved (Gollnick et al., 2007).

2.4 Pathology

2.4.1 Gross Pathology

Gross and microscopic lesions associated with JD are primarily confined to the intestine, mesenteric lymph nodes and ileo-caecal lymph nodes (Buergelt et al., 1978). Gross changes are characterised by thickening and corrugation of the intestinal mucosa. Lesions tend to be most prominent in the distal ileum and ileo-caecal valve (Buergelt et al., 1978, Clarke, 1997).
Figure 2.3. Thickened intestines with dilated serosal lymphatics.

Figure 2.4. Mucosal section showing thickened and corrugated intestines

Serosal lymphatics are often dilated and torturous (Whitlock and Buergelt, 1996). Lymph nodes maybe enlarged up to five fold, oedematous and soft (Whitlock and Buergelt, 1996). Clinical signs of diarrhoea, however, are not always correlated with the macroscopic changes (Brady et al., 2008).
2.4.2 Histopathology

Histopathology (or histology) involves the examination of sampled whole tissues under the microscope. Histological lesions associated with JD can vary widely (Clarke, 1997). In cattle there is a much greater variety of lesions compared to sheep (González et al., 2005). Based on the type of lesions observed there are two main pathological forms described paucibacillary and multibacillary (Clarke and Little, 1996). Macrophages form the main inflammatory cells in multibacillary lesions, with lymphocytes being the main inflammatory cell in paucibacillary (Clarke, 1997).

- **Diffuse multibacillary lesions** have been reported by many (Buergelt et al., 1978, Clarke, 1997). Lesions show macrophage with foamy cytoplasm and include epithelioid cells (activated macrophage) infiltrating the intestinal wall. Villi are frequently fused and the mucosa is invariably thickened. An infiltrate of macrophages with giant cells are commonly identified in the submucosa. Acid fast bacilli are commonly present. The muscular layer and serosa have multifocal granulomatous infiltrates present. Lymph nodes can show distortion of the normal architecture, due to diffuse granulomatous lymphadenitis with macrophage and giant cells in the cortex and paracortex.
In clinical cases epithelioid macrophage are plentiful in the mucosa and submucosa. The tips of the villi fuse reducing the surface available for absorbing nutrients. The granulomatous enteritis leads to a protein loosing enteropathy which manifests clinically as sub mandibular oedema (Whitlock and Buergelt, 1996).

Figure 2.5. Histopathology section from a cow confirmed to be infected with JD. The figure shows diffuse, severe blunting and fusion of villi, a diffuse granulomatous infiltrate, with a severely thickened mucosa and sub mucosa.

2.5 Diagnostics

2.5.1 Introduction

As the treatment of MAP is generally regarded as ineffective, diagnostic testing is often used to direct subsequent management decisions (e.g. calf in separate area, cull etc.) and allow preventative management of non infected herd mates. As MAP is a slow growing bacterium, infection can remain latent in infected
animals for many years without showing clinical signs, making diagnosis difficult (Nielsen, 2009a). Diagnostic tests in use involve either the identification of MAP itself (culture), identification of MAP genetic elements (PCR), or a detection of the immune response MAP infection elicits (ELISA) (Manning and Collins, 2001). Matrix-assisted laser desorption/ionization–time mass spectroscopy (MALDI-TOF MS) is an emerging tool that can be used to aid identification of bacterial isolates directly from cultures (Cai et al., 2014), and it may play an important role in JD diagnosis in the future.

The diagnostic sensitivity (Se) of a test is a mark of a test’s ability to identify the target condition if it is there i.e. true positives. Diagnostic specificity (Sp) reflects the ability of a test to give a negative result in the absence of the target condition (true negatives) (Sekiya et al., 2013). Different stages in the infection process can occur; these can be classed as infected, infectious and affected (Nielsen and Toft, 2008), with a higher level of bacterial shedding associated with affected animals. The application of diagnostics at advanced stages of the disease are usually associated with improved Se and Sp (Gillardoni et al., 2012). Nielsen and Toft (2008), report that formal comparison of tests is not justified. This is due to variation in study design and target conditions (infected, infectious, and affected). In situations where an animal is showing clinical signs compatible with Johne’s disease however, antibody tests, faecal culture, and faecal PCR will all perform with comparable sensitivity (>90%) (Sweeney et al., 2012).

Choice of diagnostic test will hinge on the speed the farmer wishes to reach disease eradication status, economic factors and initial herd prevalence (Sweeney et al., 2012). Generally the speed of eradication coincides with the cost of testing. Without commitment, however, to implementing management changes based on diagnostic test results, it is of little value to initiate costly testing schemes (Sweeney et al., 2012).

Generally control programmes incorporate an initial herd screen followed by confirmatory testing. As part of Animal Health Ireland’s (AHI) (see Section 2.8.1:
Control) pilot JD control programme, ELISA testing is used to identify suspect animals. PCR and faecal culture tests are used to confirm test status.

2.5.2 ELISA

The enzyme linked immune sorbent assay (ELISA) examines the host's immune response to MAP. ELISA is extensively used for the routine diagnosis of MAP. ELISA relies on the identification of antibodies to a particular antigen as an indicator of infection (Sekiya et al., 2013). Given the nature of the host's immune response for MAP where there is low antibody production in the subclinical stages (Stabel, 2000), accurate diagnosis can be hampered by variability in ELISA Se and Sp (Nielsen and Toft, 2008). ELISA Se ranges from approximately 15% in subclinically affected animals to over 90% in clinical cases (Olsen et al., 2002, Gilardoni et al., 2012).

ELISA positivity is correlated with the level of faecal shedding (Sweeney et al., 1995, Kalis et al., 2002). ELISA has a higher sensitivity in animals with a heavier bacterial load, i.e. high shedders compared to lower shedders (Whitlock et al., 2000). Koets et al. (2001) showed that on average, cows shedding MAP had higher concentration of MAP antibodies. Also, Collins (2002) showed cows with high level of serum antibodies to MAP had high odds of being in the advanced stages of infection. Repeat testing of herds over time will result in decreased test sensitivity, which is understandable as heavy shedders will be removed first (Whitlock et al., 2000).

MAP shares a number of antigens with other mycobacterial species (Harris and Barletta, 2001). This antigenic cross reactivity can interfere with ELISA test Sp. Preabsorption of test sera with Mycobacterium phlei is a step used to increase the specificity of ELISA tests (Collins, 1996). This step removes non-specific cross reacting antibodies (Yokomizo, 1985), however infections with MAA etc may result in antibodies that cross react with antibodies reacting to MAP (Nielsen et al., 2001, Nielsen, 2009a). In herds with a high prevalence of environmental mycobacteria, many false positive ELISA results may be obtained (Osterstock et al., 2007, Roussel et al., 2007). Diagnostic tests for bovine tuberculosis have also been reported to interfere with MAP ELISA in
seronegative herds (Vargas et al., 2009). No research has been conducted in seropositive herds. Furthermore little research has been conducted on the impact of tuberculosis testing on MAP ELISA in countries that conduct a similar level of tuberculosis testing as Ireland.

ELISA tests can be performed on both blood and milk samples. Differing reports exist in relation to the comparibility of blood and milk ELISA. Reports vary from not being significantly different (Lombard et al., 2006), moderate agreement (Hendrick et al., 2005a), to lacking correlation (Hardin and Thorne, 1996). Early lactation (Nielsen et al., 2002b) and late lactation (Lombard et al., 2006) are associated with increased ELISA positives in milk, with Nielsen and Toft (2012) reporting on the dilution effect of milk yield relating to decreased odds of testing positive for MAP. Equally stage of lactation is an important consideration for serum ELISA, with Nielsen et al., (2002b) reporting increased odds of testing positive in late lactation. First lactation cows are least likely to test positive on both blood and milk ELISA compared to older cows (Nielsen et al., 2002b). As the disease is slowly progressive, and antibody response typically does not manifest until the latter stages of the disease (Stabel et al., 2000), ELISA tests rarely give a positive result in animals under 2 years of age, and frequently fails to detect individuals in the early phases of infection (Juste et al., 2005). Despite these disadvantages, ELISA testing of sera is still the method of choice for epidemiological studies and herd-based diagnosis (Böttcher and Gangl, 2004), mainly due to it’s speed and low cost relative to other diagnostic techniques (Olsen et al., 2002).

To be compliant with AHI’s JD programme, herds must complete either one serum or two milk ELISA tests per year.

2.5.3 Culture

Faecal culture is generally taken as the reference test for MAP (Olsen et al., 2002) and is one of the confirmatory tests of choice for AHI’s control programme. An advantage of culture is that detection of MAP in faecal samples confirms the presence of viable MAP in the sample (Sweeney et al., 2012). Due
to the lack of shedding, or intermittent shedding of the bacteria early in the
disease process, the Se of culture can be low (Nielsen and Toft, 2008, Gilardoni et al., 2012), ranging from 23% to 70% in infected, infectious and affected animals (Nielsen and Toft, 2008). The specificity of culture however, is usually considered to be almost 100% (Nielsen and Toft, 2008). Evaluation of the transmission risk posed by animals can be estimated by enumeration of colony-forming units (on solid media), or time to detection (on liquid media) (Sweeney et al., 2012).

Pooling of samples for faecal culture has been associated with a reduction in Se (Kalis et al., 2000), however it may be considered as a cost-effective method of detecting MAP infection at a herd level (Wells et al., 2002, Wells et al., 2003). Targeted sampling of the environment for faecal culture can also be performed (Raizman et al., 2004), with Tavornpanich et al. (2008) reporting that six environmental culture samples is the most cost effective way of identifying if a herd is positive, however low prevalence herds can be missed. Samples collected from a high prevalence herd or highly contaminated environment that record low levels of MAP on culture, need to be interpreted with caution as these may represent “pass through” of bacteria recently consumed rather than shed from infected intestinal tissue i.e. the animal may not be truly infected (Sweeney et al., 1992b, Fecteau et al., 2010). Other disadvantages of culture are the slow speed and the cost of the technique.

2.5.4 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is used to detect DNA of MAP, and is used as a confirmatory test in AHI’s pilot JD scheme. PCR offers a rapid method of detecting MAP status (Clark Jr et al., 2008). Different genetic targets can be identified with PCR. The insertion sequence (IS900) identified by McFadden et al. (1987), is commonly used to test samples as it is sensitive and considered highly specific. Englund et al. (2002) have however, suggested other mycobacteria can harbour IS900-like sequences and the assay may not be 100% specific. While IS900 remains the favoured target sequence (Möbius et al., 2008), an alternative target element in MAP called F57 can be utilised (Möbius et al., 2008). Estimation of Se and Sp again shows wide variation.
Bayesian estimates reported include 29% and 99.3% as seen by Wells et al. (2006), and 60% and 97% by Alinovi et al. (2009). As with other MAP tests, the infection status of the animal is important in the interpretation of the disease (Wells et al., 2006). A disadvantage of PCR is the cost in comparison to other diagnostic methods such as ELISA (Olsen et al., 2002). PCR can aid however, with gathering a better understanding of epidemiology and genetic diversity. Genotyping using PCR has identified four genotypes in Ireland (INMV_1, INMV_2, INMV_3 & INMV_116) (de Kruijf, 2016).

Other lesser used ante-mortem tests available in Ireland include Interferon gamma and intradermal tests.

2.5.5 Interferon gamma

Interferon tests evaluate the production of the cytokine IFN γ by T lymphocytes during an 18–36-hour incubation period with antigen (johnin). To quantify the detection of IFN γ an ELISA is used. The overall sensitivity of this test is low (Stabel, 1996), but for animals in the subclinical stages the Se is higher than that of the serological test. An IFN γ response may occur prior to the onset of faecal shedding (Jungersen et al., 2002). This test is rarely used in Ireland, other than in a research setting (Britton, 2016).

2.5.6 Intradermal testing

Intradermal skin tests have also been used as a JD diagnostic to measure the cellular immune response in the early stages of infection (Olsen et al., 2002). Animals are intradermally inoculated with either avian purified protein derivative (PPDa) or johnin (johnin is analogous to PPD used for bTB testing (see 2.9 Tuberculosis) but made from a sterile solution of the growth products of MAP). Repeat skin thickness measurements are taken 72 hours post PPD administration. Animals showing increased skin measurements of > 2mm (OIE, 2016) are classified as positive. Both johnin and PPDa are reported to have comparable sensitivities and specificities (Gilardoni et al., 2012). A comparative test (SICCT, see Section 2.9 Tuberculosis) using both PPDa and PPDb is also in use where animals with MAP infection react at both sites, but a greater intensity of reaction is recorded at the PPDa site (Gilardoni et al., 2012). While
Manning and Collins (2001) suggest further monitoring of animals showing strong responses to PPDa with regard to MAP infection, skin testing is not recommended for JD surveillance due to its low specificity (OIE, 2016). Little work has been done on the use of intradermal testing for MAP in an Irish context. As both PPDa and PPDb are administered on at least an annual basis to all Irish cattle, potentially this represents an under utilised resource and may be of supplementary use in Irish control programmes.

2.5.7 Necropsy

Paratuberculosis cannot be diagnosed on superficial post mortem examination of the intestines alone. Diagnosis should be confirmed by the collection of multiple intestinal wall and mesenteric lymph node samples for histology (OIE, 2016). See Section 2.4 Pathology for further information.

2.5.8 Ziel- Nieslon

Stained smears of faeces or intestinal mucosa can be used as an aid to diagnosing MAP. Ziel-Nielsen staining is normally incorporated as part of post mortem examinations. The technique stains bacteria with an intact cell wall. A presumptive diagnosis can be made if clumps (3+) of strongly acid-fast bacilli are identified. An inconclusive result is reported if single acid-fast bacilli in the absence of clumps are found. Although cheap and fast, the disadvantages of this test are that it does not differentiate among mycobacterial species (OIE, 2016). Bacteria such as Nocardia and Corynebacterium may also stain acid fast (Thoresen et al., 1994, Brees et al., 2000). Sensitivity may also be poor due to the scarcity of organisms in the samples (Condron et al., 1994).
2.6 Prevalence

2.6.1 Prevalence Overview

Since the formal identification of JD in 1895, the disease has been identified worldwide in cattle populations (Chiodini, 1993, Ayele et al., 2001, Sweeney et al., 2012).

- Apparent prevalence of a disease at an animal or herd level can be defined as the number of animals or herds testing positive by a diagnostic test divided by the total number of animals or herds tested.

- True prevalence is the actual number of diseased animals/ herds divided by the number of individuals/ herds in the population. True prevalence may never really be known for a population, unless all animals in a population are tested with an assay that is 100% accurate. Estimates of the true prevalence can however be calculated from the apparent prevalence (Sergeant, 2017). The Rogan Gladen method is frequently used (Rogan and Gladen, 1978), however it is believed Bayesian inference is more flexible (Gardner, 2004). The Bayesian approach incorporates prior knowledge (Vilar et al., 2015) of the true prevalence.
and test sensitivity and specificity, based on previous data or expert knowledge. There is limited work in Ireland relating to MAP prevalence using Bayesian estimation (McAloon et al., 2016a).

When investigating disease prevalence, important considerations include choice of diagnostic test and case definition (Nielsen and Toft, 2009), as Se and Sp of diagnostic tests improve in clinical stages of the disease. Equally of note, in highly contaminated environments the prevalence of clinical cases would be expected to be higher and cases expected to occur at a younger age than in cleaner environments (Whittington and Sergeant, 2001). Due to different case definitions, diagnostic tests used and study design, comparing disease prevalence across studies proves difficult and comparable estimates can rarely be calculated (Nielsen and Toft, 2009). Best estimates from a review of prevalence across Europe estimated an animal level prevalence of approximately 20%, with herd level true prevalence estimates at >50% (Nielsen and Toft, 2009). In a study in Ireland involving over 20,000 animals, an animal level true prevalence of 2.74% in dairy herds and 3.09% in beef herds was recorded. The herd true prevalence reported for dairy and beef herds was 19.6% and 6.3% respectively (Good et al., 2009).

2.7 Economics

2.7.1 Economic Losses

Economic losses associated with JD are frequently reported at farm, national and international levels, and thus prove to be key drivers in the implementation of JD control programmes (Garcia and Shalloo, 2015). Due to the prolonged insidious nature of JD and variability in sensitivity and specificity of diagnostic tests (Nielsen and Toft, 2008), estimation of economic impacts can however be challenging. The reported economic effects can contrast greatly, with Benedictus et al. (1987) reporting economic losses may be so high that farming
cannot be profitable any more, while other studies report JD to be of lesser financial consequence than other diseases (Stott et al., 2005).

When estimating production effects it is necessary to consider, management systems in place on farm (Kömendy et al., 1988), farm size (Benedictus et al., 1987, Ott et al., 1999, Hasonova and Pavlik, 2006), diagnostic tests employed to identify JD positive animals (Johnson-Ifearulundu et al., 2000) and stage of infection. Animals in clinical stages of disease are more likely to show production effects than subclinically affected animals. Economic impact may be difficult to measure in herds with a low prevalence or with small numbers of animals in the subclinical stage (Lombard, 2011). Donat et al. (2014), investigated the effect of within-herd prevalence on the level of variation in milk yield and found a greater decline in herds with greater within-herd prevalence.

• Animal Losses
One of the greatest economic losses associated with JD is death of clinically ill animals or obtaining reduced slaughter values (Hasonova and Pavlik, 2006). If an animal is prematurely culled or dies due to JD, consideration must also be taken of loss of potentially high genetic merit animals and their future breeding potential (Hasonova and Pavlik, 2006) and an increase in herd replacement costs (Raizman et al., 2009). Loss of farm reputation is also an important consideration on farms that sell breeding stock (Hasonova and Pavlik, 2006).

• Milk Yield Losses
A reduction in milk yield is a frequently reported association with testing JD positive (Lombard et al., 2005, Gonda et al., 2007, Sorge et al., 2011, Smith et al., 2015, McAlloon et al., 2016b). In contrast a number of additional studies have identified no such association (Johnson et al., 2001, Hoogendam et al., 2009). Interestingly, higher milk production was identified by Smith et al. (2015) in positive cows prior to starting faecal shedding. This wide variability in results has led to Hutchinson, (1996) and Hasonova et al, (2006) identifying the need for further studies investigating milk production effects of JD.

• Milk Solids
In relation to milk solids Gonda et al. (2007), Donat et al. (2014) and Hendrick et al. (2005b) showed lower production of milk fat and protein in JD positive cows. Contrastingly, Lombard et al. (2005) found no significant effect of testing ELISA positive on milk solids production.

- **Fertility**
Studies relating to fertility again highlight the variability reported across studies. An increase in 'days-open' (i.e. an increased interval from calving to conception) in ELISA positive cows was reported by Johnson-Ifeareulundu et al. (2000). In contrast, Lombard et al. (2005) showed ELISA positive cows to have fewer non-pregnant days. Equally Gonda et al. (2007) showed higher pregnancy rates (1.39%) in JD infected cows. Gonda et al. (2007) suggests the ELISA response in cows with increased pregnancy rates may be evidence of a robust humoral response, and that even though MAP antibodies area considered ineffective for MAP, antibody production in general may be critical for defence against other pathogens.

- **Somatic Cell Count**
Research relating to Somatic cell count (SCC) has failed to show a consistent association between MAP test status and clinical or subclinical mastitis. Numerous studies (Lombard et al., 2005, Gonda et al., 2007, Hoogendam et al., 2009) recorded no association between MAP ELISA positivity and SCC which contrasts with other studies (Dieguez et al., 2008, Pantoja et al., 2010) that reported an associated increase in SCC in MAP ELISA positive dairy cows.

- **Negative energy balance and costs of ineffective treatment**
JD and its associated intestinal malabsorption and protein losing enteropathy (Whitlock and Buergelt, 1996) can result in decreased feed efficiency and poor weight gain in both clinical and subclinical animals (Johnson-Ifeareulundu et al., 1999) which will reduce farm profits. As negative energy balance is associated with locomotive and digestive problems (Collard et al., 2000), it is possible JD predisposes to other diseases. Moreover infection with JD has been proposed to increase the risk of secondary disease due to the impairment of cell mediated immunity (Kreeger and Snider 3rd, 1992). Bennett (2003), found higher levels of
input e.g. more veterinary inputs associated with the presence of the disease. As there is currently no effective treatment for JD, ineffective veterinary treatment for animals infected with JD should be considered as a potential economic loss (Hasonova and Pavlik, 2006).

- **Economic losses reported in Irish Studies**

In relation to Ireland contrasting reports exist relating to the economic impact JD has on farms. Richardson and More (2009), report animals showing clinical signs of JD to have a significantly lower milk yield (1259.3 kg/lactation) and an average cull price 516 euro less than clinically normal herd mates. Similarly, in a herd with clinical signs of JD, Barrett et al. (2006) reports reduced farm performance due to decreased milk yield, increased culling and reduced cull cow values. Contrastingly, in a study involving 949 cows across a number of herds, Hoogendam et al. (2009) found no statistically significant effect of testing MAP ELISA positive on milk yield, solids, fertility or SCC. This variability within Irish studies alone necessitates further Irish economic investigations using multiple herds with varying prevalence.

- **International Reports**

Although Stott et al. (2005) found JD to be of lesser financial consequence than a number of other diseases, in certain cases costs of JD at both the farm level and a national industry level are undeniably substantial (estimated to cost US dairy industry between $200- $250 million per year (Ott et al., 1999)). Should the hypothesised link between JD and Crohn’s disease in humans ever be proven however, scenario analysis of changes to dairy product consumption conducted by Groenendaal and Zagmunt (2008) shows potentially catastrophic damage to the global dairy industry, in certain scenarios costing upwards of billions of dollars, necessitating the implementation of control programmes. This is perhaps most pertinent in countries like Ireland that direct a high level of milk produced towards infant formula markets.
2.8 Control Programme

2.8.1 Introduction

JD is a notifiable disease in a number of countries including Ireland, Sweden and Austria. Due to animal welfare implications, economic losses and potential zoonotic links associated with JD, control programmes have been established at both regional and international level (Geraghty et al., 2014). Countries engaging in programmes include Australia, Canada, Denmark, the Netherlands, the United Kingdom and the United States of America (Geraghty et al., 2014). Breaking the cycle of transmission through the identification of potentially MAP infected animals via testing, and preventing transmission to other animals through management measures forms the basis of many control programmes (Chiodini et al., 1984).

A voluntary pilot national JD control programme was established in Ireland in 2013, under the guidance of Animal Health Ireland (AHI). The primary aspects of this programme involve identification of potentially infected animals via either milk/ blood ELISA testing. Confirmatory testing is based on faecal culture or PCR. A key element of AHI's scheme is veterinary risk assessment and management planning (VRAMP). Garry (2011), indicates that the initial step toward successful MAP control is to identify weaknesses in management and propose changes. AHI'S VRAMP involves interaction between a trained veterinarian and the farmer. A detailed examination of farm management practices is conducted. Biosecurity practices, calf, heifer and calving area management are all evaluated. The aim is to highlight practices that may contribute to a risk of JD transmission. Recommendations are given with annual reassessment to evaluate compliance and to suggest further plans for reducing the risk of disease transmission. A number of other countries utilise similar risk assessment plans as an aid to JD control including Canada, Denmark and the USA (Geraghty et al., 2014). To measure the success of such a programme however, baseline data of pre-programme farm management practices occurring in Ireland are required.
### 2.8.2 Management Practices

A number of practices have been identified as high risk for the transmission of JD, and as such, avoiding such practices is recommended when participating in JD control programmes (see Section 2.2.4 Risk factors). Prevention of contact between calves and adult cow faeces forms the basis of a number of suggested practices, as this contact is identified as the greatest risk factor for JD transmission (Doré et al., 2012). Additionally, Sweeney et al. (2012), provide a comprehensive list of management recommendations which include the following:

<table>
<thead>
<tr>
<th>Calving area management</th>
<th>Colostrum management</th>
<th>Management of calves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevent access of sick or test positive cows to the calving area</td>
<td>Do not pool colostrum</td>
<td>House separately from adult animals</td>
</tr>
<tr>
<td>Regularly clean and apply fresh bedding to the calving area</td>
<td>Do not use colostrum of unknown MAP status</td>
<td>Use milk replacer or pasteurised milk</td>
</tr>
<tr>
<td>Limit the level of manure on cow’s udder, flanks and legs in both the dry cow area and the calving area</td>
<td>Do not use colostrum from test positive dams</td>
<td></td>
</tr>
<tr>
<td>Remove calves from the calving area ASAP and prevent the calf suckling the dam</td>
<td>Thoroughly clean all equipment including feeding tubes buckets etc.</td>
<td></td>
</tr>
</tbody>
</table>

Success of control programmes and cost benefit analysis of certain aspects of management programmes have been evaluated. Collins et al. (2010) report
successful control of JD on farm during a six year field trial. Similar management practices to above were used, combined with culling of strong ELISA positive cows. It is important to note the implementation costs of a control program involving testing regimes may be considerable (More et al., 2015). Dorshorst et al. (2006), found however, it was economically acceptable to test cows as long as results were used to manage positive cows.

Simulation studies have shown test and cull programmes alone without closing infection routes are ineffective in reducing prevalence and are not cost-effective methods (Groenendaal and Galligan, 2003, Kudahl et al., 2008). Lu et al. (2010) found the combination of testing and culling along with improved management measures, may be more effective than utilising either strategy in isolation, while Groenendaal and Galligan (2003) found that improving calf hygiene practices were the most economically attractive control measures. A control programme examining implementation of an on farm JD control programme combining culling and improved management has yet to be conducted in an Irish context.

- **Vaccination**

The use of vaccination has been advocated in some control programmes. Use is prohibited in a number of countries including Ireland however, primarily due to interference with tests for tuberculosis (Garcia and Shalloo, 2015). There is evidence vaccination prevents progression of clinical signs although it does not reduce faecal shedding of the organism (Sweeney et al., 2012) indicating vaccination can not replace hygienic management practices.

### 2.8.3 Challenges

The type of control programme implemented will be dictated by how aggressively the producer wishes to pursue eradication. Much of this will hinge on starting prevalence, and whether income from selling replacement stock is an aim. The cost of a control programme will generally be inversely related to the speed with which eradication can be approached (Hasonova and Pavlik, 2006).
A significant challenge that has emerged in a number of programmes is counteracting the declining programme participation over time (Geraghty et al., 2014). Kudahl et al. (2008) identifies challenges with participation in control programmes, including slow progress requiring farmers to remain motivated for years while often hampered by inadequate diagnostic tests. Further to this, a number of the management recommendations are perceived as labour intensive at busy time periods (Dorshorst et al., 2006, Kudahl et al., 2008). Benefits to calf health however, due to JD control programmes have been reported (Sorge et al., 2010, Wolf et al., 2014). It may be true that in certain cases entering a JD control programme may not appear economically attractive. Simulation models predict however, that with no control programme in place economic losses will rise with increasing herd prevalence (Kudahl et al., 2008). Indeed the potential zoonotic link with Crohn’s alone, makes it incumbent on all dairy producers to minimise the levels of JD in their herds.

2.9. Tuberculosis

2.9.1 Overview

*Mycobacterium bovis* (*M. bovis*) is another member of the Mycobacteriacea family that is pathogenic and definitively zoonotic (Moda et al., 1996). It is the causative agent of bovine tuberculosis (*bTB*), a chronic disease that mainly targets the lungs and their draining lymph nodes (Domingo et al., 2014). In the late 1800’s Robert Koch identified the aetiological agent of tuberculosis and also developed tuberculin (a glycerol extract of pure culture of the tubercle bacilli). Koch recognised tuberculin’s value as a diagnostic test, giving rise to the delayed type hypersensitivity test (DTH).

Similar to MAP, *M. bovis* is an intracellular pathogen of macrophages, with the predominant immunological response being cell mediated effected by T cells (Ritacco et al., 1991). Ante mortem tests of cellular immunity therefore have
greater sensitivity for *M. bovis* than antibody mediated tests (de la Rua-Domenech et al., 2006). The tests currently approved by the EU for bTB testing, include the intradermal tuberculin test and the IFNy assay (de la Rua-Domenech et al., 2006). Purified protein derivative used in intradermal testing is a refinement of liquid culture of tubercle bacilli originally discovered by Koch (de la Rua-Domenech et al., 2006).

2.9.2 Irelands TB eradication Programme

The compulsory eradication scheme enacted in Ireland for bTB began in the 1960's, due to production losses and public health concerns associated with *M. bovis* and remains necessary to allow compliance with European trading conditions (Directive 64/432/EEC) (Good, 2006). Ireland operates a comprehensive bTB eradication scheme. The program involves ante-mortem and post-mortem diagnostics.

- Post mortem diagnosis involves the veterinary inspection of all bovine carcasses for human consumption. Examination includes palpation, incision and visual inspection of bronchial, mediastinal and prescapular lymph nodes and the lungs, with suspect lesions being sent for confirmatory testing, usually by culture.

- Ante mortem diagnosis of bTB in Ireland involves comprehensive disease surveillance using a skin test known as the single intradermal cervical comparative test (SICCT).

The SICCT involves intradermal introduction of bovine and avian purified protein derivatives (PPD) at two different sites of the neck to elicit a delayed hypersensitivity response (DTH). Comparative measurements at both injection sites, taken 72 hours post PPD administration, are used to assess infection status. A difference in reaction size of >4mm at the bovine PPD inoculation site compared to the avian site indicates a positive result (Monaghan et al., 1994). Purified protein derivative is created by growing the organism (*M. avium*/*M. bovis*) on liquid culture, heat treating, filtering, washing and re-dissolving into a
sterile preparation free from intact mycobacteria (Monaghan et al., 1994, de la Rua-Domenech et al., 2006). When injected, PPD acts as an antigen to identify an animal whose immune system has been previously sensitized by infection with *M. bovis* or by exposure to cross reacting antigens (de la Rua-Domenech et al., 2006). PPD elicits a DTH response mediated by T cells. Injected antigen is transported to regional lymph nodes where memory Th1 cells are activated in previously sensitized animals and hone back to site of injection and mediate responses (Monaghan et al., 1994).

**Figure 2.7.** Skin thickness measurements are taken at the site of PPD administration both before injection and 72 hours later to assess infection status.
A once annual test is undertaken by all herds in Ireland, with additional bTB tests of herds contiguous to or otherwise epidemiologically linked (e.g. purchased animals from a herd later identified as bTB positive) with infected herds. Identification of a reactor animal leads to the animals prompt removal to slaughter, with the implementation of additional herd bTB tests at 60 day intervals until two clear tests in succession are achieved. Test interpretation at these additional checks is more severe, with difference in reaction size of 2mm between avian and bovine PPD indicating a positive result (Good, 2006). The comprehensive nature of bTB testing in Ireland may see some animals facing up to five tests in one year (Good et al., 2007).

The Se and Sp of the bTB skin test is not perfect, with non-specific hypersensitive reactions to the test reported in animals after natural, experimental or vaccinal exposure to bacteria that share proteins with bovine PPD (de la Rua-Domenech et al., 2006). Animals infected with other species of mycobacteria generally develop a greater response to avian PPD compared to bovine PPD. The high level of environmental mycobacteria in Ireland necessitates the use of the comparative test (Cooney et al., 1997). In other countries the caudal fold test and the single intradermal test (SIT) are used which involves the administration of bovine PPD only. Programmes in New Zealand, USA, South Africa and Brazil use the caudal fold test. The cervical SIT is used as the primary test in Italy (de la Rua-Domenech et al., 2006). A number of countries also use the SICCT as a confirmatory test following a positive caudal fold test e.g. Brazil (de la Rua-Domenech et al., 2006). The testing interval can vary greatly between countries e.g. in Ireland routine SICCT takes place every 12 months, with the UK employing two separate testing regimes; mandatory annual herd testing in parts of the UK, with other herds requiring testing every four years (APHA, 2017).
2.9.3 Interferon Gamma Test

Other tests available include the IFN gamma (IFN\textsubscript{\gamma}) test. IFN\textsubscript{\gamma} test is a measure of the animals cell mediated immune response (Pollock et al., 2005). IFN\textsubscript{\gamma} is a cytokine released by T lymphocytes, following stimulation of the immune system by antigen. The IFN\textsubscript{\gamma} test involves incubation of individual blood samples in the presence of test antigens (usually PPD avian and bovine), with a second stage of the test used to quantify the amount of IFN\textsubscript{\gamma} using ELISA (Rothel et al., 1990). The IFN\textsubscript{\gamma} test identifies animals at an earlier stage of infection than the skin test (Pollock et al., 2005). Some of the disadvantages include an apparent lower test specificity compared to skin testing, higher cost and logistical difficulties with sample processing (Gormley et al., 2004b). In Ireland the IFN\textsubscript{\gamma} test is used as an adjunct to skin testing in problem herds to reduce the time to eradicate infection.

Due to the large amount of shared antigens, animals with JD can cause false positive reactions in both TB tests. Equally \textit{M. bovis} infected animals can cross react with JD ELISA tests (de la Rua-Domenech et al., 2006). Little research pertaining to the inter-relationship between \textit{M. bovis} diagnostics and MAP ELISA tests has occurred specifically in countries engaging in such a comprehensive bTB eradication programme as Ireland. Consequently the potential interference with test Sp necessitates thorough investigation.

2.10. Methods

2.10.1 Statistics

Statistics provides a summary of numerical data, models relationships between variables and allows decisions to be made on the basis of incomplete information. Statistics frequently involves studies on samples of the population with the aim of applying these results to the whole population (Winters et al., 2010). In broad terms there are two types of data, numerical data where things are directly measured e.g. blood alcohol content. Categorical data are assigned to non-numerical data e.g. gender or nationality (Winters et al., 2010). In this thesis some frequently encountered terms relating to statistics include:
Mean: this is the arrhythmic average of a set of data. It is prone to outliers.

Median: is the middle value of a set of numbers arranged in chronological order. The median is not influenced by outliers.

Distribution: refers to the shape of the curve obtained when values of data are plotted on a graph. The bell shaped curve reflects normal (Gaussian) distribution. In this distribution the mean is equal to the median (Winters et al., 2010).

Figure 2.8. Bell-shaped curve of a normal distribution and the proportion of values falling within three standard deviations of the mean.

Standard deviation denotes how far away from the mean an individual value lies. Outliers are values that fall more than 3 standard deviations from the mean. In normally distributed data 68% of observations fall within one standard deviation of the mean, 95% with in 2 and 99.7% of values fall within 3 standard deviations of the mean.
Box plots are useful for presenting data. Data is split into quartiles with 25% of the data lying below quartile 1 (Q1). Q2 represents the median below which 50% of the data lies, with 75% of the data lying below Q3. Box plots are useful for comparing two or more distributions (Williamson et al., 1989).

![Box plot diagram](image)

**Figure 2.9.** Diagram of a box plot showing minimum, median and maximum. Data is split into quartiles with 25% of the data lying below quartile 1 (Q1). Q2 represents the median below which 50% of the data lies, with 75% of the data lying below Q3.

Experimental studies involve examination of variables. Variables can be described as dependent or response variables (i.e. the outcome of the experiment), or independent or explanatory variables (i.e. variables that can influence the experimental outcome) (Festing and Altman, 2002). Variables can be continuous measurements e.g. test results, categorical groupings e.g., alive
or dead, or ordinal increments e.g. small, medium or large (Petrie and Watson, 2006).

The type of statistical test performed will depend on the distribution of the data. Parametric tests are performed on normally distributed data and are more powerful than non-parametric tests. It is more difficult to display statistical significance with non-parametric tests (Winters et al., 2010).

Sample size calculation is an important part of study design when inferences about a population are to be made. The sample size is the number of experimental units that need to be included in a study to answer a research question (Noordzij et al., 2011). It needs to be a true representation of the population. A larger sample size more closely approximates a normal distribution.

To avoid introducing bias to a study random sampling of experimental units is important (Festing and Altman, 2002). While random sampling can be as basic as choosing every second animal in a group, if the target population is expected to differ demographically e.g. differing enterprise types e.g. dairy or beef farming, in different geographical locations, stratified sampling can be used (Petrie and Watson, 2006). This involves choosing sample units proportional to the total number in each stratum of the target population. Usually a 95% confidence level and 5% confidence interval are chosen. This means that we can be 95% confident that the prevalence is X ± 5% where X is the prevalence figure generated by the study (Shannon and Walter, 1995).

A null hypothesis is a type of hypothesis or theory used in statistics that proposes that no statistical significance exists in a set of given observations. The null hypothesis attempts to show that no variation exists between variables. It is presumed to be true until statistical evidence nullifies it for an alternative hypothesis.

The probability ($P$) that the mean value of the experiment originates from the extreme tails of a normal probability plot, when that mean value originates from the reference population is known as the $\alpha$-probability. In biological experiments, $P<0.05$ is the significance level commonly used (Thrusfield, 2013). As the null hypothesis assumes that the sample mean of two groups (reference group and study group) are the same, should $P<0.05$ (i.e. the $\alpha$-probability is
extremely low), the null hypothesis is rejected and the comparison of two means is not the same. Biological significance must be differentiated from statistical significance in that the findings must be relevant to a clinical outcome (Petrie and Watson, 2006).

2.10.2 Epidemiology

Epidemiology is the study of disease, and the factors leading to its occurrence in a population. It involves analysis of patterns of disease in both time and space (Thrusfield, 2013).

The objectives of epidemiology are to:
- investigate how and where a disease originated
- control a disease when its origins are poorly understood
- gather data relating to the history of a disease
- design and assess the success of disease control programmes
- analyse the economic effects of a disease including providing information for a cost benefit analysis (Thrusfield, 2013).

2.10.3. Statistics in Epidemiology

Some commonly used statistical techniques in epidemiology include:
The chi-square statistic is used to show whether or not there is a relationship between two categorical variables. It is a non-parametric test (Winters et al., 2010).

The t-test assesses whether the means of two groups are statistically different from each other and can be used on continuous data (Winters et al., 2010).

Pearson's correlation coefficient \((r)\) is used to demonstrate whether two variables are correlated or related to each other. When using Pearson's correlation coefficient, the two variables in question must be continuous, not categorical. Correlation does not equal causation. It indicates the variables are not totally independent of each other (Winters et al., 2010). The non-parametric
counterpart of Pearson correlation is Spearman Correlation performed on ordinal data (Winters et al., 2010).

**Regression** is a powerful statistical tool as it allows you to determine the effect of one independent variable on your dependent variable while holding any number of other independent variables constant.

The most frequently used regression analysis in this thesis includes

(i) **Linear regression**: this is used to describe a linear relationships when the dependent variable is continuous. The independent variable can be continuous, dichotomous, or categorical. Simple linear regression involves examining the association between one dependent and one independent variable. Multivariable regression is applied when more than one independent variable is included in a model (Petrie and Watson, 2006).

(ii) **Logistic regression**: Logistic regression can be seen as analogous to linear regression. This is used to describe a relationship when the dependent variable is dichotomous (i.e. yes or no; diseased or not-diseased etc.). In this case linear regression is not appropriate as the residuals are not normally distributed. The outputs, odds ratios (OR) will highlight the increased or decreased odds of the dependent variable occurring given the independent variable (Petrie and Watson, 2006).

(iii) **Multinomial regression**: this is used where the dependent variable has more than two outcomes e.g. clinical outcome of disease being absent, mild, or severe. In this case, one outcome is chosen as the base outcome, and the probability of being in another category compared to the base outcome is calculated. As with logistic regression outputs are in the form of ORs (Petrie and Watson, 2006).

A commonly encountered issue in veterinary epidemiological studies is clustering of data (Carpenter, 2001). Clustered data may include clustering in space e.g. cows within herd or may include clustering in time e.g. repeated measurements on the same experimental unit i.e. the cow.
Generalised mixed models include both fixed and random effects, and can be used for clustered data, for example data with repeated observations (Goldstein et al., 2002). Mixed models are an extension of generalized linear models. Fixed-effects terms are usually the conventional linear regression part and the random effects are associated with individual experimental units drawn at random from a population. In the case of repeated measurements from a population of experimental units over time (longitudinal data), these measurements are not independent. While mixed models can be used for analysis, another option is generalised estimating equations (GEE) (Ghisletta and Spini, 2004). GEE can successfully analyse repeated data for both categorical and continuous variables. An advantage of GEE is their ability to deal with non-normal data (Ghisletta and Spini, 2004). Another advantage of GEE is its ability to deal with missing data (Hanley et al., 2003). The focus of the GEE is on estimating the average response over the population "population-averaged" effects.

Another commonly used epidemiological tool is survey taking. Surveys can be used as a foundation for discussion and for targeting education and training requirements (Brandt et al., 2008). As the information is self-declared it is important to design questions to obtain the most truthful options. Analysis of questionnaire can be done in a similar way to observational studies, however the answer to the survey questions become the dependent variable. Independent variables can include a number of predetermined parameters e.g. geographical region, herd size etc. The starting point for survey analysis by computer is the coding and entry of data. Online software is available to help with this analysis e.g. www.surveymonkey.com.

2.10.4 PCR

PCR (Polymerase Chain Reaction) is a relatively simple, inexpensive and fast technique used to amplify or copy small segments of DNA. In a PCR experiment, two primers are designed to match a segment of DNA. Through complementary base pairing, one primer attaches to the top strand at one end
of the segment of interest, and the other primer attaches to the bottom strand at the other end.

PCR requires a DNA template and a free 3′ (3 prime) end at each of the target DNA strands. The template is provided by the DNA sample to be amplified and the free 3′ are provided by site-specific primers. Primers are short pieces of laboratory manufactured DNA. They are custom built, to contain any desired sequence of nucleotides. Design of primers can be difficult as areas unique to the pathogen of interest must be identified in order to achieve specific DNA amplification. In JD IS900 is frequently targeted for PCR (McFadden et al., 1987, Douarre et al., 2010).

DNA extraction from faeces

- One gram of faeces was weighed into a 50ml Falcon tube containing 5 mL of 10 mM TE (ph8). The purpose of TE buffer is to solubilize DNA, while protecting it from degradation.
- The sample was vortexed for 1 minute and left to stand for 30 minutes.
- 800 µL of the supernatant was then transferred to 2 mL screw capped tube containing acid washed glass beads and 800 µL CTAB buffer.
- The sample is incubated at 65 °C for one hour, with gentle inversion occurring from time to time.
- The samples were sheared in a Ribolyser (MagNA Lyser, Roche) for 45 seconds at 6500 m.s⁻¹
- 800 µL of the supernatant was then transferred to a 2 mL Eppendorf tube.
- The disrupted cell extract was then mixed with an equal volume of phenol:chloroform:isoamyl alcohol 25.24.1. The samples were shaken manually for 5 minutes and centrifuged at max speed for 30 minutes at 4 °C.
- The DNA was then precipitated by adding 2 Vol of 100% ethanol and 0.5 Vol of ammonium acetate (7M) at -20°C overnight.
- The sample was then centrifuged at max speed for 30 minutes at 4 °C.
- The supernatant was then discarded and centrifuged for an additional minute to ensure all the ethanol was removed.
The pellet was left air dry for 10-15 minutes.

Once dry 50 µL of TE-buffer was added and mixed up and down by pipetting.

### qPCR amplification reactions

Amplification reactions were conducted as described by Douarre et al., (2010). Reactions were performed using a reaction mixture consisting of 2× LightCycler® 480 SYBR Green I Master. This contained FastStart Taq DNA Polymerase, reaction buffer, dNTP mix, SYBR Green I dye and MgCl2, 0.5 µM of forward and reverse primers and PCR grade water. Primer sequences for the amplification were 5’-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3’ and 5’-GGCGTTGAGGTCGATCGCCACGTGAC-3’ (reverse primer) Sample tubes contained 5 µl of DNA, extracted from the faecal sample as described above. Controls consisted of nuclease free water (negative control) and a positive control containing 1 µl of MAP DNA. Samples were run according to the following conditions: 1 cycle at 95 °C for 10 minutes and 35 cycles at 95 °C for 10 seconds, 60 °C for 10 seconds, and 72 °C for 16 seconds. All PCR positive samples were then assessed by melting curve profile and analysed by gel electrophoresis on a 2% agarose gel.

### 2.10.5 Faecal Culture

Post initiation of AHI’s pilot programme, all faecal culture samples in this study were performed by DAFM staff (Central Veterinary Research Laboratory [CVRL], Kildare, Ireland) as described by Britton et al, (2017). Faecal samples were cultured using the automated TREK ESP® para-JEM® system (ThermoScientific). Confirmation was performed using Herrolds Egg Yolk agar slopes both with and without mycobactin. The procedure was as follows:

A faeces sample weighing 2 g was placed in 35 mL sterile distilled water. This solution was then manually shaken and allowed to stand for 30 minutes. Subsequently, 5 mL was removed from the top of the solution using a Pasteur
pipette and placed into 25 mL 0.9% hexadecylpyridinium chloride (HPC) in 50% brain heart infusion broth (BHIB) for overnight incubation at 37 °C.

The next day, faecal samples were centrifuged at 3000g for 20 minutes at 15 °C. The supernatant was discarded and 1mL of an antibiotic mix from the para-JEM® Antibiotic Supplement in 50% BHIB was added to the pellet and allowed to incubate at 37 °C overnight. The following day, para-JEM® bottles were inoculated with 1mL para-JEM® Growth Supplement, 1mL para-JEM® Egg Yolk Supplement, 0.5 mL para-JEM® Antibiotic Supplement and 0.5 mL para-JEM® BLUE before the addition of 0.5 mL of each sample. The para-JEM® bottles were vortexed for 2 minutes, the top of the bottles were swabbed with 70% alcohol and the TREK connectors were attached, prior to being placed in the TREK ESP® culture system at 37 °C for a minimum of 42 days (Britton et al., 2017).

The para-JEM® bottles were removed from the automated system and shaken for 2 minutes and the TREK connector removed, before the contents were stained using the Ziel Nielsen method to identify acid fast bodies (AFB). Samples positive for AFB were subjected to PCR confirmation and cultured on Herrolds Egg Yolk agar slopes both with and without mycobactin and incubated at 37 °C for a minimum of 8 weeks (Britton et al., 2017).

In Chapter 6 (pre-AHI scheme) culture was performed using an in house method as described by Douarre et al, (2010). In brief, one gram of faeces was added to 20 mL of sterile distilled water. Samples were vortexed for 1 minute and allowed to stand for 30 minutes. Five mL of the supernatant were added to 25 mL of 0.9% hexadecylpyridinium chloride and left undisturbed overnight at room temperature. Tubes were centrifuged at 1700g (4300 rpm) for 20 minutes. The supernatant was removed and the pellet was re-suspended in 1 mL of 50 μg/ml amphotericin B. HEYM agar containing vancomycin, nalidixic acid and amphotericin B at 50 μg/mL were inoculated with 0.2 mL of the suspension and incubated in sealed 25 cm³ tissue culture flasks at 37 °C for 24 weeks.
2.10.6 ELISA Technique

Engvall and Perlmann first introduced the ELISA in 1971 (Engvall and Perlmann, 1971). ELISA is an immune assay that relies on the identification of antibodies to a particular antigen as an indicator of infection (Sekiya et al., 2013). ELISA is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. In an ELISA technique antibodies in samples need to attach to a specific antigen (Dowall et al., 2012). An antigen is any substance which is capable of inducing a specific immune response (Lanzavecchia, 1985). An ELISA utilises antibodies to capture an antigen and an enzyme labelled antibody to estimate the amount of the antigen (Kemeny and Challacombe, 1988, Crowther, 2001).

The usefulness of ELISA is due to the recognition process of antibody for antigen being highly specific, and antibodies will rarely cross-react, with a compound unrelated to its eliciting antigen. The binding of antibody to antigen is primarily due to the shape of the antibody-binding site. The site is complementary to that of its corresponding antigen (Wingren and Hansson, 1997).

![Antigen Binding site](image)

**Figure 2.10** The antibody binding site is a complementary shape to that of its corresponding antigen

62
Different forms of the procedure are in use but all follow the same general principle of adsorption of the antigen to a solid phase, addition of a primary (1°) antibody (Ab) which may be fluorescently, or enzyme labelled. If the 1°Ab is not labelled, a 1°Ab-specific labelled secondary (2°) Ab (e.g. goat-anti-rabbit HRP) is added, and finally an enzyme substrate which yields a signal that can be recorded is added. A number of the commercially available ELISAs (IDvet kit, Idexx) available for MAP are indirect ELISAs.

For consistency in results, all ELISA tests performed in this study were conducted using the ID Screen Paratuberculosis Indirect Screening test (IDvet). The test was conducted as follows:

- In a 96-well pre-dilution microplate, samples and controls were diluted in Dilution Buffer 6. Dilution Buffer 6 is a neutralizing buffer containing M. phlei to avoid cross reactions.
- Samples were then incubated for between 5-45 minutes at 21°C.
- 100µL of the neutralised samples and controls were transferred to the ELISA microplates (which are coated with purified extract of MAP).
- The plates were incubated for 45 minutes at 21°C (short incubation) or overnight (16-20 hours) at 5°C.
- The wells were emptied and washed three times with approximately 300 µL of wash solution. Care was taken to avoid drying of wells between washings.
- The conjugate was prepared by diluting the concentrated conjugate to 1/10 (short incubation) or to 1/25 (overnight incubation) in Dilution Buffer 3.
- 100µL of this conjugate was added to each well and the plates were covered and incubated for 30 minutes at 21°C.
- The wells were then emptied and washed three times with approximately 300 µL of wash solution.
- 100 µL of the substrate solution was added to each well and incubated for 15 minutes at 21°C.
• To stop the reaction 100 μL of Stop Solution was added.
• Optical Density was recorded at 450 nm. Results were reported as sample to positive ratio (S/P ratio) calculated using the formula 
\[ S/P \text{ ratio} = \left( \frac{(\text{OD Sample} - \text{OD Negative control})}{(\text{OD Positive control} - \text{OD Negative control})} \right) \times 100. \]
Chapter 3: A survey of management practices on Irish dairy farms with emphasis on risk factors for Johne's disease transmission

DOI: 10.1186/s13620-014-0027-9
3.1 Abstract

Johne's disease (JD) is a chronic granulomatous enteritis affecting ruminants. A number of farm management practices are associated with increased risk of JD transmission. The aim of the current study was to document JD-related management practices currently employed on Irish dairy farms. Survey questions focused on calving area (CA), calf and manure management. Independent variables (region, calving-season, enterprise type, herd size and biosecurity status) were used to examine influences on JD associated dependent variables (survey questions). Additionally general biosecurity practices were also examined.

Results showed management practices implemented by Irish dairy farmers pose a high risk of JD transmission. Of the farmers surveyed, 97% used the CA for more than one calving, 73.5% and 87.8% pooled colostrum and milk respectively, 33.7% never cleaned the CA between calving's, and 56.6% used the CA for isolating sick cows. Survey results also highlighted that larger herds were more likely to engage in high risk practices for JD transmission, such as pooling colostrum (OR 4.8) and overcrowding the CA (OR 7.8). Larger herds were also less likely than smaller herds to clean the CA (OR 0.28), a practice also considered of risk in JD transmission.

Many management practices associated with risk of JD transmission were commonly applied on Irish dairy farms. Larger herds were more likely to engage in high risk practices for JD transmission. Control programmes should incorporate educational tools outlining the pathogenesis and transmission of JD to highlight the risks associated with implementing certain management practices with regard to JD transmission.
3.2 Introduction

Johne's disease (JD), a chronic granulomatous enteritis of ruminants, is caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Ayele et al., 2001). Significant economic losses have been reported on cattle farms due to infection with MAP. Such losses are primarily due to decreased slaughter value (Raizman et al., 2009), reductions in milk production in dairy cows (Chi et al., 2002), sub-optimal fertility (Johnson-lfearulundu et al., 2000), and an increase in cow replacement costs (Ott et al., 1999). The impact of JD on animal health and on-farm profitability has led to considerable interest in the control of MAP at farm level. Controlling JD however proves difficult due to the variable progression from sub-clinical to clinical stages of disease, combined with diagnostic difficulties especially in the early stages of infection (Whitlock and Buergelt, 1996). As test and cull programmes alone prove largely ineffective in eradicating MAP from a herd (Kudahl et al., 2008), incorporation of improved calf management practices, including calf-related hygiene, may prove of more benefit in reducing on farm prevalence (Groenendaal and Galligan, 2003).

Infection with MAP predominantly occurs in calves, with animals less than six months of age being most susceptible (Windsor and Whittington, 2010). The severity and rate of JD progression in individual animals are dependent on the MAP exposure dose and the age of the animal at infection (Sweeney, 1996). Infection usually occurs via the faecal-oral route, although in-utero transmission can occur (Seitz et al., 1989). Doré et al. (2012), concluded that exposure of calves to adult faeces is the most important risk factor in MAP transmission. Faecal-oral transmission is facilitated by faecal contamination of a calf's environment and feedstuffs, with the primary environmental risk factors for neonatal infection being faecal contamination of the udder or calving pens (Lombard, 2011). Colostrum and milk from infected cows can also contain quantities of MAP capable of infecting calves (Nielsen et al., 2008),(Streeter et al., 1995). Feeding of pooled colostrum from multiple cows (Nielsen et al., 2008), and feeding milk containing antibiotic residues (milk obtained from cows undergoing antibiotic treatment such as for mastitis) to calves (Ridge et al.,
are also both considered to increase the risk of MAP infection within a herd.

Additional management-related risk factors for MAP transmission include group housing of periparturient cows (Wells and Wagner, 2000), the presence of more than one cow in a calving pen (Tiwari et al., 2009), use of group calving pens (Pithua et al., 2013), faecal contamination of udders of periparturient cows (Ansari-Lari et al., 2009), and use of maternity pens that are not cleaned between each calving (Johnson-Ifeearulundu and Kaneene, 1998). Larger sized herds (Wells and Wagner, 2000, Muskens et al., 2003, Hirst et al., 2004, Scott et al., 2006), are associated with an increased risk of testing MAP ELISA positive. Allowing young-stock access to pasture contaminated with adult manure can also be considered a risk factor due to the prolonged survival of MAP in slurry (Jorgensen, 1977). Finally, biosecurity (Mee et al., 2012, Sayers et al., 2012), is an essential component of disease prevention in general, and is equally important in the prevention of JD, with purchase of animals considered a significant route of MAP transmission between farms (Künzler et al., 2014).

Concern has been raised regarding the zoonotic potential of MAP (Dalziel, 1913) a potential link between MAP and Crohn’s disease in humans having been postulated. Proof of a causal link would have important consequences for the global dairy industry (Groenendaal and Zagmutt, 2008). The possible public health implications of MAP make it incumbent on milk producing nations to minimise the risk of consumers ingesting MAP contaminated milk. The most recent estimate of JD herd exposure prevalence in Irish cattle is approximately 20% (Good et al., 2009), which compares favourably with estimates in other European countries (Nielsen and Toft, 2009). Additionally, between the years of 1995 and 2002 only 232 clinically infected animals (an average of approximately 30 animals per year in a cattle population of approximately six million) were detected by the Irish Department of Agriculture, Food and the Marine laboratories (DAFM) (Barrett et al., 2006). Although a relatively low prevalence is reported, the dairy industry plays a critical role in Ireland’s economy (More, 2009) and as such a JD pilot control programme has been embarked upon to further reduce the levels of MAP in Irish cattle. This Animal
Health Ireland (AHI) (More et al., 2010) co-ordinated programme uses risk assessment and management plans (RAMPs) as an integral part of the scheme (www.animalhealthireland.ie). These risk assessments involve evaluation of four key JD risk areas namely, management of pre-weaned heifers, management of heifers to first calving, mature cow environment and hygiene, and management of the calving area.

Investigations into herd demographics (Richardson et al., 2008) and risk factors associated with introduction and transmission of JD and testing JD positive on Irish dairy farms have previously been conducted (Cashman et al., 2008),(Barrett et al., 2011). The risk factors identified in these studies included larger herd size (Barrett et al., 2011), importation of cattle from abroad (Richardson et al., 2008, Barrett et al., 2011), and not using individual calving pens (Cashman et al., 2008). These findings are in agreement with the international studies described previously. Although risk factors for testing positive for MAP have been identified in Ireland, a national survey documenting the prevalence of application of JD risk-associated management practices at farm level has not previously been reported. Such a study may highlight underlying reasons for Ireland’s relatively low prevalence of JD test positive individuals and herds. The aim of the current study, therefore, was to document utilisation of management factors associated with JD transmission on Irish dairy farms, based on both national and international risk data, using a geographically representative group of Irish dairy farms. This will provide a baseline for JD risk in Ireland, which can subsequently be used to allow targeting of specific management practices that require improvement as part of control programmes. Key influences on the application of JD-associated management factors were also investigated.

3.3 Materials and methods

3.3.1 Survey Procedure

The survey was conducted as a postal survey with survey packs containing a cover letter, a self-addressed envelope, and a questionnaire, mailed to
participants for completion and return. The study population included farmers that participated in a larger disease prevalence study, the selection of whom has previously been outlined by O'Doherty et al. (2013). In brief, 500 randomly selected members of HerdPlus® (a breeding management decision support tool co-ordinated by the Irish Cattle Breeding Federation [ICBF]) were invited to participate. Selection was based on stratified proportional sampling using strata of herd size and geographical location. A total of 312 herds elected to participate in the study with participation entirely voluntary and non-incentivised. The study population has previously been shown to be geographically representative of Irish dairy herds (O'Doherty et al., 2013). The overall project was approved by the Moorepark ethics committee in November 2008.

3.3.2 Survey Questionnaire

Questions were compiled based on information gathered from peer-reviewed publications, a commercially available web-based herd-health management tool (www.myhealthyherd.com), and Teagasc researcher experience of Irish dairying systems. Following consultation with researchers at the Animal and Grassland Research and Innovation Centre, Teagasc (Irish Agriculture and Food Development Authority) and piloting of the questionnaire by farm managers based at seven Teagasc research farms, a number of minor modifications were made to the questionnaire prior to circulation to study participants. The final questionnaire consisted of an initial section containing 17 JD-associated questions (Table 3.1) and a second section containing a further 30 questions examining general bioexclusion and biocontainment (collectively referred to as biosecurity) management practices (Figure 3.1). Johne's disease associated questions related to the calving-area (CA) and CA hygiene, milk and colostrum management, and access of young calves and in-calf heifers to adult faeces. These survey questions (dependent variables) were presented in a closed format with three response options offered, namely Yes (Y), No (N), or Sometimes (S). A subset of the population (approximately 10%) was re-surveyed in order to quantify the Sometimes responses. Where Sometimes was
chosen as an answer, an extra closed question was asked with the options of either A= <50% of the time or B= >50% of the time offered (Table 3.1). Biosecurity-related questions were again presented as closed questions offering Yes and No binary responses.
Table 3.1: JD Questionnaire responses

<table>
<thead>
<tr>
<th>Que.</th>
<th>Management Variable</th>
<th>n</th>
<th>Response</th>
<th>Outcome (%)</th>
<th>If Sometimes What % of the time?*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calving area (CA) management</td>
<td></td>
<td></td>
<td></td>
<td>A: Less than 50% B: More than 50%</td>
</tr>
<tr>
<td>1</td>
<td>Is the CA frequently used for more than one calving at any one time?</td>
<td>303</td>
<td>No</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sometimes</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Is the CA overcrowded? (e.g. more than five cows in calving pen at any one time)</td>
<td>302</td>
<td>No</td>
<td>57.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sometimes</td>
<td>29.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>13.2</td>
<td>X</td>
</tr>
<tr>
<td>3</td>
<td>Is the CA cleaned out between every calving and bedded with clean dry bedding?</td>
<td>300</td>
<td>No</td>
<td>33.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sometimes</td>
<td>37</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>29.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Is the CA used to house sick cows?</td>
<td>297</td>
<td>No</td>
<td>42.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sometimes</td>
<td>54.9</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Do cows have manure soiled legs and udders?</td>
<td>300</td>
<td>No</td>
<td>43.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sometimes</td>
<td>51</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calf feeding management</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Do new born calves stay with mother in CA for more than six hours?</td>
<td>304</td>
<td>No</td>
<td>17.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sometimes</td>
<td>39.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>43.4</td>
<td>X</td>
</tr>
<tr>
<td>7</td>
<td>Is the calf allowed to suckle from the cow?</td>
<td>303</td>
<td>No</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sometimes</td>
<td>32.7</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>60.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Is colostrum collected without disinfection of the teats prior to collection?</td>
<td>301</td>
<td>No</td>
<td>23.3</td>
<td>50:50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sometimes</td>
<td>25.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>50.8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Are heifer replacement calves fed with pooled</td>
<td>302</td>
<td>No</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Question</td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Sometimes</td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------------------------------------------</td>
<td>---</td>
<td>------</td>
<td>-----</td>
<td>-----------</td>
</tr>
<tr>
<td>10</td>
<td>Are heifer replacement calves fed pooled surplus milk from healthy cows?</td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Sometimes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>45.7</td>
<td>12.2</td>
<td>25.1</td>
</tr>
<tr>
<td>11</td>
<td>Are heifer replacement calves fed milk from sick and mastitic cows?</td>
<td></td>
<td>62.7</td>
<td>40.5</td>
<td>33.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Manure management</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Is milk and feed area for calves contaminated with cow manure?</td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Sometimes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.7</td>
<td>88.7</td>
<td>10.6</td>
</tr>
<tr>
<td>13</td>
<td>Do calves have direct contact with cows and their manure prior to weaning?</td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Sometimes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.7</td>
<td>78.3</td>
<td>13</td>
</tr>
<tr>
<td>14</td>
<td>Do calves have access to pasture which has had cow slurry applied in the same season?</td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Sometimes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18.5</td>
<td>27.8</td>
<td>53.6</td>
</tr>
<tr>
<td>15</td>
<td>Do heifers have direct contact with cows and their manure prior to entering milking herd?</td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Sometimes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37.4</td>
<td>21.9</td>
<td>40.7</td>
</tr>
<tr>
<td>16</td>
<td>Is water and feed area for heifers contaminated with cow manure?</td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Sometimes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9.9</td>
<td>75.2</td>
<td>14.9</td>
</tr>
<tr>
<td>17</td>
<td>Do heifers have access to pasture which has had cow slurry applied in the same season?</td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Sometimes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30.6</td>
<td>11.5</td>
<td>57.9</td>
</tr>
</tbody>
</table>

*A subset of the population was re-surveyed to quantify the Sometimes responses. X indicates the response chosen by the majority of the subpopulation, 50:50 indicating an equal number choosing A or B.*
3.3.3 Descriptive Analysis and Herd Classification

Hardcopy survey responses were entered into an online survey software package (www.surveymonkey.com) with electronic inputs being manually checked against hardcopy versions. Coded responses to each question were subsequently downloaded and Microsoft Excel (MS Office, Version 2010) used to organise the data, fix variables for directionality, and complete descriptive analysis.

Questionnaires were deemed suitable for analysis if greater than two thirds of survey questions were answered. Based on Irish Central Statistics Office (C.S.O., 2012) data, study herds were assigned to two geographical regions i.e. dairy dense (southern region) and non-dense (northern region), with herd calving-season categorised as spring-calving (i.e. ≥85% of the herd calved between January and March) and non-spring-calving (calving at other times of year). The livestock enterprise type was classified as dairy only or mixed-species livestock herds (i.e. herds that also contained beef cattle and/or sheep), with herd size categorised as small (31 to 65 cows), medium (66 to 99 cows), or large (>99 cows). The bioexclusion classification of each herd i.e. open (free movement of new purchases onto the farm) or closed (no introduction of new purchases onto the farm) was available from a parallel study as were a number of additional management factors (O'Doherty et al., 2013).

3.3.4 Statistical Analysis

Chi-squared, logistic regression and correlation (Pearson and Spearman) analyses were completed using Stata data analysis and statistical software (Version 12). Prior to statistical analysis an initial model was created with ‘sometimes’ response options excluded. This allowed direct comparison between those answering definitively ‘yes’ or ‘no’ (Model 1). In the interest of completeness, survey response options were also dichotomised yielding two further datasets for analysis i.e. Model 2 = Y+S versus N and Model 3 = Y versus S + N. A total of five herd classification independent variables (i.e. region, calving-season, enterprise type, herd size, bioexclusion status) were
used to examine key influences on JD risk variables. As a first step, a univariable (Pearson's Chi-squared) analysis was completed. Independent variables recording $P \leq 0.15$ were included in logistic regression models (1, 2 and 3). A manual backwards elimination with a forward step was applied to each model with significant variables ($P \leq 0.05$ chosen as accepted significance level) retained in the final model. Pearson's correlation was used to check for co-linearity across independent variables. Spearman's rank correlation ($r_s$) was performed to examine relationships between dependent variables (JD survey questions) with $r_s$ values of $>0.3$ reported. Biosecurity variables were not statistically analysed.

3.4 Results

3.4.1 JD Questionnaire Descriptive and Statistical Analysis

Of the 312 surveys returned, a total of 306 farmers returned JD questionnaires suitable for analysis yielding a 98% completion rate. Following exclusion of six questionnaires as incomplete, non-responders for individual JD questions ranged from two to nine participants. The majority of study herds (67%) were located in the dairy dense region of Ireland. Of the participating herds, 27% were categorised as small, 31% as medium, and 42% as large herd size. Similar to the national trend in the Republic of Ireland (Graham et al., 2013),(ICBF, 2014), spring-calving systems were operated by 87% of study herds, with 52% operating mixed livestock enterprises. A total of 54 herds (17.7%) were categorised as closed (O'Doherty et al., 2013). As results from Model 1 (Table 3.2) represented farmers that were definitive in responding either Yes or No to survey questions, this model is discussed in detail. Comparable associations, however, were observed in all three Models.
### Table 3.2: Significant associations between independent and dependant variables

(Model 1: Yes versus No)

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>Odds Ratio</th>
<th>P Value</th>
<th>Conf. Interval (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Is the CA overcrowded?</td>
<td>&gt;99 cows vs. 66-99 cows</td>
<td>5.0</td>
<td>0.001</td>
<td>1.9, 13.0</td>
</tr>
<tr>
<td></td>
<td>&gt;99 cows vs. 31-65 cows</td>
<td>7.8</td>
<td>&lt;0.001</td>
<td>2.6, 23.6</td>
</tr>
<tr>
<td>Do new born calves stay in CA for more than six hours?</td>
<td>31-65 cows vs. &gt;99 cows</td>
<td>3.1</td>
<td>0.009</td>
<td>0.1, 0.8</td>
</tr>
<tr>
<td></td>
<td>Non-spring &gt;99 cows vs. spring 31-65 cows</td>
<td>28.1</td>
<td>0.016</td>
<td>1.9, 421.4</td>
</tr>
<tr>
<td>Is the CA cleaned and bedded between every calving?</td>
<td>31-65 cows vs. &gt;99 cows</td>
<td>3.6</td>
<td>0.001</td>
<td>0.1, 0.6</td>
</tr>
<tr>
<td>Are heifer calves fed pooled colostrum?</td>
<td>66-99 cows vs. 31-65 cows</td>
<td>2.2</td>
<td>0.039</td>
<td>1.0, 4.5</td>
</tr>
<tr>
<td></td>
<td>&gt;99 cows vs. 31-65 cows</td>
<td>4.8</td>
<td>&lt;0.001</td>
<td>2.3, 9.9</td>
</tr>
<tr>
<td></td>
<td>&gt;99 cows vs. 66-99 cows</td>
<td>2.2</td>
<td>0.024</td>
<td>1.1, 4.4</td>
</tr>
<tr>
<td>Is colostrum collected without teat disinfection?</td>
<td>Mixed enterprise vs. Dairy only</td>
<td>1.8</td>
<td>0.049</td>
<td>0.3, 0.9</td>
</tr>
<tr>
<td>Are heifer calves fed waste milk from sick cows?</td>
<td>Mixed enterprise vs. Dairy only</td>
<td>2.2</td>
<td>0.009</td>
<td>1.2, 3.9</td>
</tr>
<tr>
<td>Do calves have direct contact with cows/ manure pre entering milking herd?</td>
<td>Non-dairy dense vs. Dairy dense</td>
<td>2.5</td>
<td>0.034</td>
<td>0.2, 0.9</td>
</tr>
<tr>
<td>Do heifers have direct contact with cows/ manure pre entering milking herd?</td>
<td>&gt;99 cows vs. 66-99 cows</td>
<td>2.5</td>
<td>0.019</td>
<td>1.2, 5.2</td>
</tr>
<tr>
<td>Do heifers have access to pasture spread with cow slurry?</td>
<td>&gt;99 cows vs. 66-99 cows</td>
<td>7.5</td>
<td>0.028</td>
<td>0.1, 0.9</td>
</tr>
</tbody>
</table>

P Value: Significant P <0.05. CA: calving area

### 3.4.4.1 Calving Area Variables

Only 3% of study farms avoided frequent use of the CA for more than one calving at a time. Overcrowding of the CA, on at least an occasional basis, was reported by over 40% of respondents (having five or more cows in the CA at any one time was cited in the questionnaire as an example of overcrowding following questionnaire piloting). Larger sized herds were more likely to overcrowd the CA compared to small (OR 7.8) or medium sized (OR 5.0) herds. Over two thirds of the participating farmers did not clean and bed the CA.
between calvings. Smaller herds however were more likely to engage in cleaning and bedding of the CA (OR 3.6) compared to larger herds. Sick cows were housed in the calving area by over half of all respondents.

### 3.4.4.2 New-born Calf Management

Over 80% of farmers allowed a calf to remain with its dam in the CA for longer than six hours. Large non-spring calving herds were more likely than small spring calving herds to allow this to occur (OR 28.1). Smaller herds, however, were more likely than larger herds to allow calves to remain in the CA for longer than six hours (OR 3.1). Unsurprisingly, a relatively strong relationship was highlighted between time spent in the CA area and allowing the calf to suckle the dam ($r_s 0.5$), although the vast majority of farmers (90%) allowed the calf to suckle the dam regardless of the amount of time spent together.

Approximately 70% of respondents pooled colostrum for feeding calves and almost 90% pooled milk for the same purpose. Larger herds were more likely to pool colostrum than smaller (OR 4.8) or medium sized herds (OR 2.2). Feeding calves with milk from sick or mastitic cows (waste milk) was practiced in almost 60% of herds on at least an occasional basis. This was more likely to occur in mixed enterprise herds as opposed to dairy only herds (OR 2.2). Mixed enterprise herds were also more likely to collect colostrum without teat disinfection (OR 1.8). Relationships existed between those farmers pooling milk and pooling colostrum ($r_s 0.5$), those feeding waste milk to calves and pooling milk for calf feeds ($r_s 0.4$), and also between those feeding waste milk and those pooling colostrum ($r_s 0.3$) (Table 3.3).
Table 3.3: Spearman correlation values between dependent variables

<table>
<thead>
<tr>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>Q5</th>
<th>Q6</th>
<th>Q7</th>
<th>Q8</th>
<th>Q9</th>
<th>Q10</th>
<th>Q11</th>
<th>Q12</th>
<th>Q13</th>
<th>Q14</th>
<th>Q15</th>
<th>Q16</th>
<th>Q17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q2</td>
<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td>0</td>
<td>-0.3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q4</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q5</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q6</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q7</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q8</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q9</td>
<td>0</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q10</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q11</td>
<td>0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q12</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q13</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q14</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q15</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q16</td>
<td>0</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q17</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Correlations > 0.3 in bold. See Table 1 for entire list of questions.
3.4.3 Hygiene and Faeces Management

Direct access between young calves and adult cows or their manure was prevented by the majority of survey participants (78.3%), however farms in non-dairy dense regions were over twice more likely to allow contact to occur (OR 2.5) With regard to replacement heifers (>12 months), 78.1% of farmers allowed at least occasional direct heifer-cow contact to occur. Larger herds were more likely than medium sized herds to allow this heifer-cow contact to occur (OR 2.5). Over 70% of herds allowed young calves access to pasture which had slurry applied in the same grazing season, with almost 90% of participants allowing replacement heifers access to slurried pastures. Again larger herds were more likely than medium sized herds to allow heifers access to such pasture (OR 7.5). A positive correlation existed between those herds allowing access of calves and heifers to potentially contaminated pastures ($r_s$ 0.5). The majority of those surveyed prevented faecal contamination (from adult cows) of both young calf and replacement heifer feed areas and water troughs (88.7% and 75.2% respectively).

3.4.2 Biosecurity Questionnaire Descriptive Analysis

A total of 312 participants returned valid biosecurity questionnaires. Almost all study participants reported regularly inspecting farm boundaries (97.4%), with the majority also preventing access to watercourses passing through neighbouring farms (69.4%). Cattle trailers, water troughs, and oral drenching equipment were regularly cleaned by a large proportion of farmers, 81.7%, 74%, and 86.2% respectively. While almost 80% of farmers prevented mixing of different farm livestock species, less than 10% prevented nose to nose contact between different management age groups (i.e. cows, heifers, calves) on farm. The majority of farmers reported daily cleaning of walkways and collecting yards and also annual disinfection of all cattle housing. Only 36.2%, however, reported cleaning individual calf pens between successive calves. Isolation of sick animals was reported by nearly 90%, and in general, importation of colostrum and various manure types was avoided with over 90% of study
farmers not engaging in such practices. Additional biosecurity practices are included in Figure 3.1 in order of the number of farmers implementing each measure.
Figure 3.1 Responses to Biosecurity Questionnaire. The level of implementation of biosecurity practices are listed in descending order from the 12 o’clock position (n = 312).
3.5 Discussion

Closure of transmission routes (Kudahl et al., 2007) and improved calf management (Groenendaal and Galligan, 2003) are essential elements of MAP control at farm level. The aim of this survey was to document JD-related management practices utilised on Irish dairy farms, thereby identifying target areas for improvement in future studies and control programmes. Questions were designed to highlight management practices that have been associated with a risk of MAP transmission in the literature. In general it was found that management practices currently being implemented by Irish dairy farmers pose a high risk of MAP infection, with larger herd sizes more likely to engage in hazardous practices for MAP transmission.

Previous international studies reported an increased risk of MAP transmission in herds where more than one cow was allowed in the calving area (Wells and Wagner, 2000, Tiwari et al., 2009), and in herds that do not routinely clean the CA between calvings (Johnson-Ifeareulundu and Kaneene, 1998). The Irish system of dairy production is an extensive, pasture-based system, with cows grazing grass outdoors for prolonged periods of lactation (Drennan et al., 2005). This combined with a relatively low average herd size compared to other countries (ICBF, 2014), (MacDonald et al., 2007, DairyNZ, 2012), might be expected to lead to a less intensive calving system with minimal CA overcrowding and good hygiene. The results presented in the current study, however, highlight that this system does not necessarily lead to optimal CA management. Pasture-based systems must operate within the constraints of the grass-growing season, and as such, a highly seasonal calving pattern is adopted (Dillon et al., 1995, Graham et al., 2013). As compact-calving herds only experience approximately one month of concentrated calving (ICBF, 2014), it is possible that Irish farmers invest in the infrastructural capacity to deal with herd average calving rate, as opposed to maximal calving rate, leading to overcrowding of the CA at certain times of the calving season. The sub-optimal levels of CA cleaning between calvings, and the CAs frequent use, is also potentially reflective of inadequate time and infrastructural resources provided
to manage the period of intensive calving in spring. The fact that larger herds are less likely than smaller herds to clean (OR 0.27), and more likely to overcrowd (OR 7.8) the CA provides further support for this theory, with larger herds having more intensive calving seasons. The seasonal calving system operated in Ireland, therefore, could potentially lead to increased transmission of MAP by bringing about sub-optimal management of the CA. Education is therefore required to highlight the importance of optimal calving management, and availability of adequate resources (especially at peak calving season), and its contributing role in achieving effective control of JD.

Regarding use of the CA for isolation and treatment of sick cows, the proportion of farmers engaging in this practice in Ireland is similar to that reported internationally (approximately 50%) (Wells, 2000, USDA, 2008, Vasseur et al., 2010). This may again reflect increased efficiencies being sought by farmers through assigning multiple uses to existing farm infrastructure. While dual use of the CA (for both calving and hospitalisation) may be considered optimal usage of this infrastructural resource, it is placing herd-cohorts at undue risk of pathogen exposure (Fossler et al., 2005). Indeed, Norton et al. (2009) highlighted an increased risk of MAP incidence in a herd when calves are raised in an area used for cow hospitalisation. As calves are born with naive immune systems (Chase et al., 2008), use of the CA for cow hospitalisation does not present a rational use of farm infrastructure in regard to disease prevention and control.

Additional management practices commonly utilised on dairy farms to achieve greater resource efficiency include pooling of colostrum, pooling milk, and use of waste milk as a calf feed (Moore et al., 2009). Colostrum is pooled to potentially provide passive immunity from vaccinated cows (Saif et al., 1983, Fernandez et al., 1998) and to ease availability of adequate volumes of colostrum during periods of peak calving, with pooling of milk facilitating group feeding of calves. Pooling of calf feeds are highly attractive for farmers in terms of resource efficiency which may account for their extensive use on Irish farms. Additionally, Gleeson et al. (2008) showed that calf management labour-saving practises were more likely to be used as herd size increases. It is perhaps not
surprising, therefore, that large (OR 4.8) and medium (OR 2.2) sized herds in the current study were more likely to engage in the practice of pooling colostrum compared to smaller sized herds. This may also underpin the widely acknowledged increased risk of larger herds testing positive for MAP (Muskens et al., 2003, Barrett et al., 2011) as pooling of both colostrum and milk is also associated with increased risk of MAP transmission within a herd (Berghaus et al., 2005, Nielsen et al., 2008).

Waste milk may be perceived as a useful resource on dairy farms, with farmers reluctant to discard it. Waste milk can be regarded as a cost saving measure rather than using saleable milk or milk replacer as calf feeds (Moore et al., 2009). Although such feed management practices may be perceived as being resource efficient, feeding of waste milk has been associated with risk of exposure to a number of pathogens (Selim and Cullor, 1997), including MAP (Ridge et al., 2005). More specifically, a univariate analysis completed by Barrett et al. (2011) examining risk factors for testing faecal culture MAP positive, found a significant association between pooling colostrum, feeding waste milk and testing MAP culture positive. The practice of feeding waste milk is not unique to Irish dairy farmers, however, with the current study recording a slightly lower prevalence of this practice compared to UK and Australian farmers (Wraight et al., 2000, Brunton et al., 2012). The correlation ($r_s$0.3) between farmers in the current study that use the CA for housing sick animals, and feed waste milk to heifer calves, again supports a trend amongst farmers in seeking, and using, resource efficient management methods regardless of potential disease consequences. A balance therefore needs to be sought and promoted amongst farmers to allow practical and cost-efficient rearing of dairy calves without increasing exposure to potential harmful pathogens.

Opinions of veterinary experts and practitioners reported by Sayers et al. (2014) highlights avoiding slurry importation, the up keep of farm boundaries and maintaining accurate disease records as key elements in farm biosecurity, all of which the majority of the current study participants conducted, indicating a level
of good biosecurity practice implementation on farm. Veterinary experts however, ranked farmer understanding of a disease second only to maintenance of a closed herd when promoting optimum farm biosecurity (Sayers et al., 2014). While many of the JD-associated management practices used on farm appear to be resource/efficiency driven, their implementation may be due to a lack of fundamental understanding of the JD risk involved when adopting certain practices. Sayers et al. (2012) have reported farmers acknowledge the importance of biosecurity, but that lack of information may prevent improvement of biosecurity practices. The findings of the present study highlights the importance of ensuring farmers evaluate labour and cost saving management routines prior to their introduction on farms and are fully educated regarding potential disease transmission risks associated with such efficiencies. As this study has identified comparable management practices reported in international studies, the opportunity exists to examine how countries with more established control programmes tackled similar management issues to help limit MAP transmission.

A possible weakness of the current study is the use of self-reported responses, as evidenced by 43.3% of those surveyed reporting cows not to have visible faeces on the legs or udders. This weakness indeed highlights the need for independent on farm risk evaluation. The VRAMP by AHI now provides such an independent verification and will prove extremely useful in tracking the progress of Ireland’s JD control programme. In general, however, it can be concluded from this current study that a high proportion of Irish dairy farmers are engaging in practices associated with increased risk of MAP transmission. Based on existing studies, however, the prevalence of JD in Ireland, compares favourably with other milk producing nations (Good et al., 2009),(Manning and Collins, 2001, Nielsen and Toft, 2009). The relatively small size of Irish dairy herds (average herd size 60 cows), compared to other intensive dairy systems (e.g. average herd size US:120 cows; average herd size New Zealand: 393 cows) (MacDonald et al., 2007; DairyNZ, 2012, ICBF, 2014) may contribute to the lower recorded prevalence, larger herds being at higher risk of contracting JD (Barrett et al., 2011),(Muskens et al., 2003, Scott et al., 2006). As Irish farmers
intend to expand their dairy herds post-2015 due to the abolition of milk quotas (restriction on milk production) (Dillon, 2011), the overall risk of contracting JD in Ireland may increase. Additionally, as it is unlikely that all expanding herds will achieve required cow numbers within the breeding capacity of their own herds, purchase of dairy stock is likely to increase further. With an already low level of closed herds operating in Ireland currently, a further increase in the purchase and movement of livestock may exacerbate the risk of MAP transmission (Künzler et al., 2014). Positively, however, Sayers et al. (2012) have highlighted that Irish dairy farmers with larger herds are more likely to voluntarily join a health scheme, making establishment of AHI's JD programme a timely intervention.

3.6 Conclusions

Many management practices associated with risk of MAP transmission were commonly applied on Irish dairy farms. Larger herds were more likely to engage in high risk practices for JD transmission. Control programmes should incorporate educational tools outlining the pathogenesis and transmission of MAP to highlight the risks associated with implementing certain labour-saving measures with regard to JD transmission. Programmes would also benefit from promoting evaluation of management practices, for impacts on disease control, prior to their introduction on-farm.
Chapter 4: Analysis of Johne's disease ELISA status and associated performance parameters in Irish dairy cows

BMC Veterinary Research (2016) 12:43

4.1 Abstract

Infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has been associated with reductions in milk production in dairy cows and suboptimal fertility. The aim of this study was to highlight the production losses associated with testing MAP ELISA positive in Irish dairy cows. Secondary objectives included investigation of risk factors associated with testing MAP ELISA positive. A survey of management practices on study farms was also conducted, with examination of associations between management practices and herd MAP status.

Blood samples were collected from 4188 breeding animals on 22 farms. Samples were ELISA tested using the ID Screen Paratuberculosis Indirect Screening Test. Production parameters examined included milk yield, milk fat, milk protein, somatic cell count, and calving interval. The association between MAP ELISA status and production data was investigated using multi-level mixed models. Logistic regression was used to identify risk factors for testing JD blood ELISA positive at individual cow level and to identify associations between farm management practices and herd MAP status. Data were available for 3528 cows. The apparent prevalence recorded was 7.4%. Mixed model analysis revealed no statistically significant association between testing MAP ELISA positive and dairy cow production parameters. Risk factors associated with testing positive included larger sized herds being over twice more likely to test positive than smaller herds (OR 2.4 P= <0.001). Friesians were less likely to test positive relative to other breeds. A number of study farmers were engaged in high risk management practices e.g. 73.1% pooled colostrum and 84.6% of study farmers used the calving area to house sick animals throughout the year. No significant associations however, were identified between farm management practices and herd MAP status.

No production losses were identified; however an apparent prevalence of 7.4% was recorded. With the abolition of EU milk quotas herd size in Ireland is expanding, as herds included in this study were larger than the national average, results may be indicative of future JD levels if no JD control programmes are implemented to minimise transmission.
4.2 Introduction

Clinical and sub-clinical manifestations of disease can result in reductions in animal productivity leading to reduced farm profits (Fourichon et al., 1999, Chi et al., 2002). Cost-benefit analyses are often conducted to highlight these economic losses in order to promote the use of disease control schemes (Stott et al., 2005). Johne’s disease (JD) is a chronic granulomatous enteritis of ruminants and is caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Ayele et al., 2001). Infection with MAP has been associated with production losses at farm level although equivocal results are reported (Hasonova and Pavlik, 2006). Due to a prolonged subclinical phase, variable disease progression and immune response (Stabel, 1998), diagnosis of MAP infection is challenging. Enzyme linked immunosorbant assay (ELISA), is a popular method of testing for MAP due to its speed and low cost (Gilardoni et al., 2012). Despite variable sensitivity and specificity (Olsen et al., 2002) (Nielsen and Toft, 2008), ELISA testing is often the method of choice for epidemiological studies and herd-based diagnosis (Böttcher and Gangl, 2004), and forms the basis of a number of international control programmes (Nielsen, 2007). It is also a common diagnostic tool used in economic studies of JD (Hasonova and Pavlik, 2006).

Economic losses reported due to infection with MAP include decreased slaughter value (Raizman et al., 2009), reductions in milk production in dairy cows (Chi et al., 2002, Richardson and More, 2009), sub-optimal fertility (Johnson-lfearulundu et al., 2000), and an increase in cow replacement costs (Ott et al., 1999). Although losses in clinically affected animals are well defined (Ott et al., 1999, Barrett et al., 2006), losses due to subclinical infection appear less well characterised (Hasonova and Pavlik, 2006). An association between subclinical MAP infection and decreased milk yield (MY) has been identified in a number of studies (Benedictus et al., 1987, Gonda et al., 2007, Sorge et al., 2011). In contrast a number of additional studies have identified no such association (McNab et al., 1991, Johnson et al., 2001, Hoogendam et al., 2009). Similarly, conflicting reports exist regarding an association between subclinical
MAP infection and milk fat (MF) or milk protein (MP) content (Benedictus et al., 1987, Johnson et al., 2001, Lombard et al., 2005). An increased interval from calving to conception in ELISA positive cows has also been reported (Johnson-Ifeearulundu et al., 2000) in contrast to a different study showing ELISA positive cows to have fewer non-pregnant days (Lombard et al., 2005). Similarly, Lombard et al. (2005) recorded no association between MAP ELISA positivity and somatic cell count (SCC) which again conflicts with other studies (McNab et al., 1991) (Dieguez et al., 2008) that reported an associated increase in SCC in MAP ELISA positive dairy cows.

The variability that exists across diagnostic test methods may, in part, explain the conflicting performance-related data reported across various studies (Hendrick et al., 2005a, Hasonova and Pavlik, 2006). Geographical location, choice of sample matrix, size of study population, cow breeds, and positive cow classification also differs across studies. Serum samples were used for diagnostic purposes in some studies (Lombard et al., 2005, Hoogendam et al., 2009), while others used individual milk samples (Sorge et al., 2011). Gonda et al. (2007), defined a JD positive cow on the basis of serum ELISA and/or faecal culture results. Study sample sizes ranged from less than 1000 (Hoogendam et al., 2009) to 35,591 dairy cows (Sorge et al., 2011) with other studies only examining a single cow breed (Gonda et al., 2007, Hoogendam et al., 2009). In this regard, it is important that data continue to be generated on similar cow populations, using similar study designs to improve the degree of confidence that exists in the likely impact on production in MAP positive dairy cows.

The prevalence of JD is believed to be increasing in the Republic of Ireland over the last ten years (Good et al., 2009, McAloon et al., 2016). In order to prevent further increases in MAP infection, improvements in control are required on Irish dairy farms. Although a previous Irish study (Hoogendam et al., 2009) identified no significant effect of MAP sero-status on herd performance in 2004-2005, the increasing prevalence of MAP may now be impacting on Irish dairy production. The dairy landscape in Europe is changing due to the abolition of EU milk
quotas in 2015 (Dillon, 2011) and Irish farmers have been expanding herds over the past number of years (Sayers et al., 2013). Given Ireland's increasing herd size, a known risk factor for testing MAP positive (Barrett et al., 2011), this study aimed to investigate the current impact of MAP ELISA sero-positivity on individual cow milk production, SCC and calving interval. Secondary objectives included investigation of risk factors (e.g. breed, parity, calf and calving management) associated with testing MAP ELISA positive in Irish dairy herds and investigating the strength of correlation between milk and serum ELISA results.
4.3 Materials and methods

4.3.1 Study Population and Sampling

Blood samples were collected from all breeding animals aged over two years on 22 Irish dairy farms in 2012. This was conducted under licence from the Department of Health and Children, the licencing authority in Ireland at the time of the study. The location of study herds is included in Figure 4.1. All but two herds were located in the dairy dense province of Munster, Ireland (south-western region) with an additional herd in each of Ulster and Leinster. A milk sample was also collected from each cow blood sampled on 17 of these farms. All study animals were observed by a veterinary surgeon during sampling visits and none displayed overt clinical signs of JD.

Figure 4.1. Map showing location of study farms. The majority of study farms were located in the dairy dense province of Munster, with one farm located in both Leinster and Ulster
4.3.2 Sample Testing

Serum and milk samples were tested by a commercial ISO accredited laboratory (Enfer Labs, Kildare, Ireland) using the ID Screen Paratuberculosis Indirect Screening Test (ID Vet, Montpellier, France). This ELISA has a reported sensitivity (Se) of 41.5% and specificity (Sp) of 99.42 (Fry et al., 2008). The test is an *M. phlei* absorbed ELISA detecting anti-MAP immunoglobulin G (IgG). This ELISA was chosen as it is approved for use in Ireland’s national voluntary JD pilot control programme (www.animalhealthireland.ie) and displayed the highest overall accuracy of four commercial ELISA kits investigated by ROC analysis (Fry et al., 2008). Results were reported as sample to positive ratio (S/P ratio) calculated using the formula $S/P \text{ ratio} = \left( \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Negative control}}}{\text{OD}_{\text{Positive control}} - \text{OD}_{\text{Negative control}}} \right) \times 100$. Animals were assigned MAP status (positive or negative) according to kit manufacturer interpretation (‘kit-interpretation’), with serum results of $S/P \geq 70\%$ classified as positive. A more severe test interpretation was also applied to blood ELISA results only in order to achieve increased test sensitivity, similar to the revised ELISA cut-off used by Collins et al. (2005). In this current study, instead of arbitrarily choosing a more sensitive positive cut-off, the mean S/P ratio of cows classified as negative (i.e. $S/P < 70\%$) plus three times the standard deviation was used. This yielded a new ‘severe interpretation’ positive cut off of $S/P \geq 51.59$.

4.3.3 Individual Cow Performance Data

Production data for each individual cow were downloaded from the Irish Cattle Breeding Federation (ICBF) database. This database holds production data on milk recording herds to support breeding decisions and enhance genetic gain in Irish dairy herds. Production parameters downloaded included 305 day (Olori and Galesloot, 1999) Milk Yield (MY), Milk Fat (MF), Milk Protein (MP), somatic cell count (SCC), and calving interval (CI). Only calving intervals of greater than 250 and less than 500 days were retained for analysis to allow comparison with Hoogendam et al. (2009). Additional cow related data downloaded from the ICBF database included parity, breed, and economic breeding index (EBI; a
profit index measured in Euro, aimed at identifying the most profitable cows for breeding dairy herd replacements (Berry et al., 2007)).

4.3.4 Survey Data

Data relating to farm management practices on study farms were collected during scheduled farm visits. Questions focused on management practices which have previously been identified in the literature as being associated with a high risk of MAP transmission.

4.3.5 Dataset Construction

The production performance for each cow was matched to her ELISA result to create the dataset of statistical analysis. Both milk and blood ELISA results were included where available and the 'kit-interpretation' used to classify cows as positive and negative. A further dataset was constructed in an effort to identify production losses experienced by cows recording ELISA results near the manufacturers' cut off point. This dataset was constructed using the 'severe-interpretation' ELISA positive cut-off. Both blood data sets were used for the mixed model analysis with only the 'kit-interpretation' dataset used for prevalence investigations and investigating the correlation between blood and milk ELISA results. To investigate the correlation between paired milk and serum ELISA results, Spearman correlation (rs) was performed on categorical results i.e. cows classified as positive or negative.

4.3.6 Data Analysis

Data manipulation and graphical representations including box plots were completed in Excel (MS 2010). Generalised linear latent and mixed models (gllamm), logistic regression, and Spearman correlations were performed using Stata (Version 12).
4.3.7 Prevalence Calculation

The apparent prevalence's (Ap) at animal level and within herd level were calculated as total number of test positive animals out of the total number of animals tested. To estimate the true prevalence (Tp) of positive animals, a Bayesian approach and Gibbs sampling method was applied using an online epidemiological calculator (Epitools) (Sergeant, 2015). This calculator requires prior estimates of the true prevalence and test sensitivity and specificity, based on previous data or expert knowledge. These estimates are made as beta probability distributions, with parameters alpha and beta. Alpha and beta can be calculated provided estimates of the mode and 5% or 95% confidence limits are available from expert opinion. When the mode was between 0.5 and 1, the 95th percentile was entered into the Beta distribution utilities calculator, and the 5th percentile entered if between 0.5 and 1. Prior estimates for true prevalence were based on a national survey which reported a prevalence of 3.3% from a study population of 15,558 animals aged over two years (Good et al., 2009). Beta probability distributions for ELISA Se and Sp were compiled based on estimates from peer-reviewed literature (Collins et al., 2005, Fry et al., 2008), expert opinion from Irish veterinary practitioners, veterinary officers, Teagasc veterinary and agricultural researchers, and a comprehensive longitudinal farm study involving ELISA screening and confirmatory testing using faecal culture and post-mortem examination. The estimates and beta distributions used to determine Tp are outlined in Table 4.1. Upper and lower confidence limits for Tp calculation were set at 97.5% and 2.5% respectively. Outputs reported in this study are median values of posterior distributions for prevalence, sensitivity and specificity.
Table 4.1. Estimates for use in true prevalence calculation. The online epidemiological calculator (Epitools) requires prior estimates of the true prevalence and test sensitivity and specificity, based on previous data or expert knowledge. These estimates are made as Beta probability distributions, with parameters alpha and beta. Alpha and beta can be calculated provided estimates of the mode and 5% or 95% confidence limits are available from expert opinion.

<table>
<thead>
<tr>
<th>Estimates of posterior distributions</th>
<th>Beta distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alpha</td>
</tr>
<tr>
<td>Animal</td>
<td>0.033 (0.072)*</td>
</tr>
<tr>
<td>Prevalence</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.41 (0.587)*</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.99 (0.47)*</td>
</tr>
</tbody>
</table>

Initial values represent the mode, with the value in brackets representing either the 5th or 95th percentile. *When the estimated value was between 0 and 0.5 the 95th percentile was chosen, and when the estimate was between 0.5 and 1 the 5th percentile was chosen.

4.3.8 Associations between cow performance and MAP ELISA status

All data were visually assessed for normality using ladder of powers histograms in Stata. The association between MAP ELISA status and production data was investigated using gllamm. Models accounted for random effects of cow nested within herd. Covariates were retained in the final multivariable models on the basis of the highest reduction in the Bayesian information criterion (BIC). Covariates examined in each model were breed (Friesian/Friesian crosses (FRx), Jersey/Jersey crosses (JEx), Norwegian Red/Norwegian Red crosses (Redx), other), parity (1, 2, 3, 4, 5, 6, 7, 8, 9, ≥10), EBI (categorised into quartiles, category 1 being the highest quartile and category 4 the lowest) and herd size (≤150 cows, >150 cows). This number was chosen as average herd size in the study.
size of study herds was 153 cows. Second-level interactions between covariates were also examined. Values of $P < 0.05$ were considered significant.

4.3.9 Associations between breed, herd size, parity, EBI and MAP ELISA status

Logistic regression was used to identify risk factors for testing MAP blood ELISA positive at individual cow level (dependent variable). Independent variables examined in regression included breed, herd size, parity and EBI. These variables were coded as described for gllamm models. Herd of origin was forced into all models. A manual backwards elimination with a forward step was performed for each model. Interactions between variables were also examined. Variables recording a significance level of $P < 0.05$ were retained in the model and are reported.

4.3.10 Associations between farm management practices and herd MAP status

Logistic regression was used to identify associations between testing MAP blood ELISA positive at a herd level and farm management practices (survey responses). A herd was classified as positive if a minimum of one blood ELISA positive result was identified. Herd size was included in the model as a covariate, as larger herds are more likely to test positive (Barrett et al., 2011). Again a manual backwards elimination was performed with interactions between variables examined. Variables recording $P < 0.05$ were considered significant.

4.4 Results

4.4.1 Descriptive Data

Samples were collected from 4188 breeding animals. Production data were available for 3528 dairy cows, the remainder ($n=660$) being excluded from
further analysis. These exclusions consisted of misidentified animals, breeding bulls, and beef cows in herds having mixed beef and dairy enterprises. Of the 22 herds, nine contained >150 cows.

The predominant breed sampled was FRx (82.7%), the remaining 10.3%, 5.5%, and 1.5% being Redx, Jersey, and other, respectively (Figure 4.2). The majority of animals tested were parity 1 (30.1%) or parity 2 (26.4%) (Figure 4.3).

Figure 4.2. Proportion of animals tested belonging to each breed. The predominant breed tested using both milk and blood ELISA was Friesian. FRx: Friesian, JX: Jersey, Red: Norwegian red
Figure 4.3. Proportion of animals tested belonging to each parity. The majority of animals tested were of Parity 1 or Parity 2. P = Parity
Table 4.2: Section A shows the proportion of animals that were tested (either positive or negative) belonging to each breed and parity. Section B shows the proportion of animals testing positive belonging to each breed and parity.

<table>
<thead>
<tr>
<th>A</th>
<th>Total tested (n)</th>
<th>Parity 1 %</th>
<th>P 2 %</th>
<th>P 3 %</th>
<th>P 4 %</th>
<th>P 5 %</th>
<th>FRx %</th>
<th>Jx %</th>
<th>Redx %</th>
<th>Other breeds %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3528</td>
<td>30.1%</td>
<td>26.4</td>
<td>14.9</td>
<td>13</td>
<td>15.6</td>
<td>82.7</td>
<td>5.5</td>
<td>10.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Milk</td>
<td>1686</td>
<td>30%</td>
<td>22.8</td>
<td>14.7</td>
<td>13</td>
<td>19.5</td>
<td>80.5</td>
<td>3.7</td>
<td>14.1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Total Positive(n)</th>
<th>Parity 1 %</th>
<th>P 2 %</th>
<th>P 3 %</th>
<th>P 4 %</th>
<th>P 5 %</th>
<th>FRx %</th>
<th>Jx%</th>
<th>Redx%</th>
<th>Other breeds %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>261</td>
<td>29.5%</td>
<td>28.7</td>
<td>14.2</td>
<td>10.0</td>
<td>17.6</td>
<td>53.6</td>
<td>2.4</td>
<td>39.8</td>
<td>4.2</td>
</tr>
<tr>
<td>Milk</td>
<td>131</td>
<td>27.5%</td>
<td>16.8</td>
<td>13.7</td>
<td>13</td>
<td>29</td>
<td>56.4</td>
<td>3.9</td>
<td>32.8</td>
<td>6.9</td>
</tr>
</tbody>
</table>

P = Parity, FRx = Friesian, Jx = Jersey, Redx = Norwegian
Figure 4.4. Box plot showing range of S/P ratios across all herds that recorded at least one positive animal. Over half the animals in herd 1 tested positive.
4.4.2 Prevalence

The highest within herd Ap recorded on any single farm was 56%. Box plots showing range of S/P ratios across all herds with at least one positive animal are shown in Figure 4.4. The overall study Ap recorded utilising blood MAP ELISA was 7.4%. Based on the Bayesian analysis, Tp was estimated at 3.8%, with a median output test Se and Sp of 41.6% and 94%, respectively. All cows tested in three herds recorded negative blood MAP ELISA results and these herds contained <150 cows. A breakdown of the positives across parity, breed, and herd size are outlined in Table 4.2. The highest proportion of animals testing ELISA positive were third parity and Redx being proportionally the predominant breed testing positive (Figures 4.5 and 4.6, respectively), although the majority of Redx testing positive were from a single herd. Many of the animals testing positive in this herd were born in the same year.

4.4.3 Milk ELISA Results

A total of 131 from 1696 cows available for analysis, tested milk ELISA positive for MAP. Of these, 61 also tested positive on blood. The remaining 70 animals recording positive milk ELISA results tested negative on blood ELISA. It should be noted that samples from 47 ‘milk positive blood negative’ cows were collected in September/ October/ November. These samples would therefore have been collected during late lactation in the Irish Spring calving dairy system. Further to this a number of the blood results, although classified as negative, were approaching the manufacturer cut off point of 70 S/P, possibly explaining the discrepancy between milk and blood ELISA. Spearman correlation yielded an $r_s$ value of 0.19 indicating poor correlation between milk and blood test results at a categorical level (Figure 4.7).
Figure 4.5. Proportion of animals testing positive per parity. The highest proportions of animals testing blood ELISA positive were of parity 3.

Figure 4.6: Proportion of positive results recorded across each breed. Norwegian reds were proportionally the predominant breed testing positive. The majority of this breed testing positive however originated from the same herd.

Legend: FRx: Friesian, JX: Jersey, Red: Norwegian red
Figure 4.7. Scatter plot showing the relationship between matched blood and milk samples. An $R^2$ value of 0.1908 was obtained.

4.4.4 Management Practices Survey

The majority (73.1%) of respondents purchased animals onto their farms. Approximately, three quarters of study farmers fed calves pooled colostrum (73.1%) and pooled milk (76.9%). Additionally, milk not fit for sale i.e. milk containing antibiotic residues or milk from sick/mastitic cows was used to feed calves on 65.2% of study farms. Group calving pens were used by 54% of study farmers. The majority (84.6%) of study farmers also used the calving area to house sick animals throughout the year. A high proportion of farmers removed calves from the calving area within 30 minutes of birth (65.4%). Similarly, the majority of study farmers did not allow calves to suckle the dam (65.4%) (Figure 4.8)
Figure 4.8. Responses to survey questions. The questions focus on management practices that have previously been associated with JD transmission. The high risk practices for JD transmission are shown in red.
### 4.4.5 Production Data

Mean milk yield was 5494 kgs. Mean milk fat and protein was 240kgs and 196kgs respectively. As a percentage of yields this equated to 4.4% milk fat and 3.6% protein.

### 4.4.6 Mixed model analysis of production parameters

Multilevel mixed model analysis revealed no statistically significant association between testing MAP ELISA positive and MY, milk solids, CI, and SCC. Similarly, analysis of the ‘severe interpretation’ dataset again revealed no statistically significant differences (Table 4.3). No statistically significant association between testing milk ELISA positive and MY, milk solids, CI, and SCC was identified.

#### Table 4.3: Results from multilevel mixed model analysis. No significant associations between production parameters and sero status were identified utilising the manufacture cut of point of 70 or the severe interpretation cut off point of 51.59

<table>
<thead>
<tr>
<th>Dataset Name</th>
<th>Coefficient: JD positive vs. JD negative</th>
<th>Std Error</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk Kgs</td>
<td>-8.7</td>
<td>47.3</td>
<td>0.854</td>
</tr>
<tr>
<td>Protein %</td>
<td>0.14</td>
<td>0.01</td>
<td>0.424</td>
</tr>
<tr>
<td>Fat %</td>
<td>-0.03</td>
<td>0.03</td>
<td>0.343</td>
</tr>
<tr>
<td>Calving Interval</td>
<td>-3.2</td>
<td>2.0</td>
<td>0.098</td>
</tr>
<tr>
<td>SCC</td>
<td>10.3</td>
<td>21.7</td>
<td>0.635</td>
</tr>
<tr>
<td><strong>Severe Interpretation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk Kgs</td>
<td>-3.91</td>
<td>52.5</td>
<td>0.9406</td>
</tr>
<tr>
<td>Protein %</td>
<td>-0.01</td>
<td>0.01</td>
<td>0.6745</td>
</tr>
<tr>
<td>Fat %</td>
<td>-0.02</td>
<td>0.03</td>
<td>0.5257</td>
</tr>
<tr>
<td>Calving Interval</td>
<td>-4.52</td>
<td>5.04</td>
<td>0.3695</td>
</tr>
<tr>
<td>SCC</td>
<td>10.4</td>
<td>22.4</td>
<td>0.6432</td>
</tr>
</tbody>
</table>
4.4.7 Associations between breed, herd size, parity, EBI and MAP ELISA status

Statistically significant results of logistic regression analysis are included in Table 4.4. Larger herds were over twice more likely to test positive than smaller herds. Redx and breeds classed as other were more likely to test JD ELISA positive than FRx (OR 6.5, 5.5 respectively). No significant associations were highlighted between parity, EBI and MAP ELISA status.

4.4.8 Associations between farm management practices and herd MAP status

No significant association was identified between calving area, calf feeding management practices and MAP ELISA status.
**Table 4.4:** Logistic regression: Significant associations between testing MAP ELISA positive and independent variables. Larger sized herds were more likely to test positive compared to smaller sized herds. Friesians were less likely to test positive relative to other breeds examined.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Odds Ratio</th>
<th>P Value</th>
<th>Conf. Interval (95%)</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Independent Variable</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Johne's disease ELISA positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd size &gt;150 cows vs. herd size &lt; 150 cows</td>
<td>2.4</td>
<td>&lt;0.001</td>
<td>1.7, 3.4</td>
<td></td>
</tr>
<tr>
<td>Breeds</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red x vs. FRx</td>
<td>6.5</td>
<td>&lt;0.001</td>
<td>4.8, 8.9</td>
<td>Breed</td>
</tr>
<tr>
<td>Other vs. FRx</td>
<td>5.5</td>
<td>&lt;0.001</td>
<td>2.5, 12.2</td>
<td>Herd size</td>
</tr>
<tr>
<td>Red x vs. Jex</td>
<td>12.2</td>
<td>&lt;0.001</td>
<td>5.2, 28.6</td>
<td>Parity</td>
</tr>
<tr>
<td>Other vs. Jex</td>
<td>10.3</td>
<td>&lt;0.001</td>
<td>3.3, 32.1</td>
<td>EBI</td>
</tr>
</tbody>
</table>

*P Value: Significant P <0.05. Only significant results shown.*  
*<sup>a</sup> Outlines the independent variables included in the logistic regression model.*
4.5 Discussion

Economic losses are often reported due to JD (Garcia and Shalloo, 2015). Given Ireland’s increasing herd size, a known risk factor for testing MAP positive (Barrett et al., 2011), this study aimed to investigate the current impact of MAP ELISA sero-positivity on individual cow performance. Secondary objectives included investigation of risk factors (e.g. breed, parity, calf and calving management) associated with testing MAP ELISA positive in Irish dairy herds and investigating the strength of correlation between milk and serum ELISA results.

Two previous Irish reports have highlighted significant losses in JD clinically affected animals (Barrett et al., 2006, Richardson and More, 2009), but are limited to individual farm case studies as opposed to across farm studies. An additional Irish study involving 34 herds, however, reported no statistical effect of JD sero-status (sub-clinical cows) on Irish dairy cow milk and fertility performance parameters (Hoogendam et al., 2009). As the study conducted by Hoogendam et al. (2009), involved a sample size of 949 and only 11 serologically positive individuals, it was necessary to conduct a larger study in Irish dairy herds. Both the overall sample size and number of ELISA positive animals detected, were considerably higher in the current study (3528 cows and 261 positives) which would greatly increase the confidence in the findings reported. It was unexpected, therefore, that a continuing lack of statistically significant association between JD sero-positivity and performance in Irish dairy cows was highlighted. This is not particular to Ireland; with additional international studies reporting similar findings (Johnson et al., 2001, Hasonova and Pavlik, 2006). It may be suggested, therefore, that use of the ELISA tests that are currently available is not an ideal study design in order to detect sub-clinical losses due to JD infection. Studies, however, have detected losses in sero-positive individuals (Lombard et al., 2005, Sorge et al., 2011) and as ELISA testing forms an integral part of many international control programmes (Benedictus et al., 2000, Nielsen, 2007), studies aimed at highlighting production losses associated with JD ELISA status are important additions to
the global JD database. Further such studies may allow an analysis of geographical differences in the impact of sub-clinical JD across various countries, which in turn could assist in identifying protective or stimulatory factors with regard to JD infection.

The impact on production associated with testing MAP ELISA positive will differ depending on choice of diagnostic test. This is because the accuracy of MAP testing differs across both kits and stage of infection in the animal tested (Hasonova and Pavlik, 2006). Indeed the poor correlation between the use of milk and blood samples identified in the current study serves to clearly highlight the considerable variability between diagnostic test methods, especially at different stages in lactation as highlighted in this study and others (Lombard et al., 2006, Kennedy et al., 2014a). Indeed as the specificity of MAP ELISA is not 100% (Nielsen and Toft, 2008), it is possible that a proportion of the animals identified as positive in the current study may be false positives. The Bayesian estimates of Tp would support this in that it indicates a lower level of true infection than the apparent prevalence recorded. Identification of false positives and false negatives due to less than optimal test sensitivity and specificity would lead to mis-categorisation of individuals hence reducing the likelihood of detecting association between performance and ELISA status. This again stresses the importance of using large sample size studies for these analyses. The current study strove to correct for poor test sensitivity by using a 'severe-interpretation' yet production differences remained unidentifiable.

A number of environmental mycobacteria have been identified in Ireland (Cooney et al., 1997). Environmental mycobacteria are known to contribute to false positive ELISA results (Osterstock et al., 2007), potentially allowing interference with results in the current study. As faecal culture positive cows have been shown to have consistently larger effects on all production traits compared to MAP ELISA positive cows (Gonda et al., 2007), a limit of this study is the lack of faecal culture. A study conducted in the same region as the current study however, showed moderate agreement between ELISA testing and culture techniques (Cashman et al., 2008), indicating there is a level of agreement between ELISA and culture tests in the region. As, however, results
from this study indicate no economic effect of testing ELISA positive, it may indicate that future economic investigations in Ireland should not be conducted utilising ELISA alone. It may also prove worthwhile in future studies to increase the ELISA test kit cut-off as a means of potentially improving test specificity.

It was important to identify whether farmers engaged in good Johne’s management in this study to examine the influence of the farmer as a protective influence. Not only were farmers in the current study not engaging in protective practices, a higher level of adoption of certain high risk management practices than previously reported in Ireland was recorded. The current study reported 65% of study farmers feeding waste / antibiotic milk to calves compared to 59.6% in the previous national study (Kennedy et al., 2014b). Use of the calving area to house sick animals was also reported at a much higher level than previous national (Kennedy et al., 2014b) and international studies (Vasseur et al., 2010). Results from this study also show higher usage of group calving pens than that reported elsewhere (Cashman et al., 2008, Vasseur et al., 2010) possibly placing study farms at increased risk of the incidence of diarrhoea, including salmonella and JD (Losingter et al., 1995, Pithua et al., 2013). Indeed maintenance of a closed herd is a key element of general herd biosecurity (Van Winden et al., 2005) however over 70% of study farmer’s reported not operating a closed herd. Given the high level of high risk management practices reported in the current study and a previous study (Kennedy et al., 2014b) it is important to provide information to farmers about the pathogenesis and transmission of JD to minimise future JD levels. As almost all participants were utilising high risk management practices, it is perhaps unsurprising that no significant differences were identified between management practices adopted on test positive and test negative herds.

Although a high number of MAP ELISA positive animals were identified, no animal was observed to be displaying clinical signs of JD at the time of sampling. As typical results in relation to JD risk factors were identified i.e. larger herds being more likely to test positive, perhaps it indicates an unidentified element exists within Irish dairying systems that limits the production effects and clinical signs on Irish cattle. Potential protective effects
may include the widespread use of grass based systems in Ireland or the lower average herd size compared to other countries (C.S.O., 2012, DairyNZ, 2012). It may also however relate to the level of environmental mycobacteria present and the extensive TB testing programme that operates in Ireland. Infection with MAP can lead to false positive reactions on the intradermal skin test for bTB (de la Rua-Domenech et al., 2006). As a bTB test is administered on at least a single occasion annually to every bovine in Ireland, removal of ‘MAP reactors’ prior to the development of clinical signs is possible, potentially explaining negligible losses.

The annual culling rate (21%) (Maher et al., 2008) is lower than some countries. However, as opposed to many countries that operate a year round calving system, Ireland targets a seasonal breeding and calving period (Graham et al., 2013). Animals have a limited period of time in which they can be bred, and are culled at the end of lactation if shown to not be in calf. This level of culling therefore may have led to the removal of some MAP infected animals prior to the onset of clinical signs. Currently the dairying sector in Ireland and the EU in general is entering a period of change due to the abolition of EU milk quota restrictions. The Irish dairy sector has set targets to increase dairy output by 50% by 2020 (Food Harvest 2020). In order to meet this 2020 target, there is a requirement to increase cow numbers by 350,000 in Ireland by 2020, compared to average cow numbers between 2007 and 2009 (Shalloo et al., 2012). With the abolition of EU milk quotas and the intent of many farms to expand in size (Dillon, 2011), it may be that animals that would previously have been culled may in future be retained in the herd, potentially leading to an increased risk of MAP transmission. As this study involves herds larger than the national average herd size and results show an increased number of animals testing positive than previous Irish studies, it will be important to monitor the production effects associated with testing JD positive, as national herd size expands.

The greater likelihood of Redx to test positive relative to JEx and FRx was a surprising result from this study as there is a low level of JD in the breed’s country of origin and also due to a speculated increased resistance to JD in
Norwegian red cattle (Holstad et al., 2005). Norwegian Reds are commonly utilised in breeding programmes to increase herd genetic merit (Buckley et al., 2008). In the 1990's a large number of dairy cattle were imported to Ireland from continental Europe (Barrett et al., 2006). The practice of importing cattle from abroad has been associated with increased risk of testing JD positive (Barrett et al., 2011). It is possible that farms in the present study that utilised Norwegian Reds to improve herd genetic merit may have previously imported cows from abroad, possibly allowing the establishment of JD within the herd, facilitating current transmission to Norwegian Red cows. Indeed it is possible that herds interested in cross breeding and improving herd genetic merit may be more progressive and potentially larger in size further exacerbating the increased odds of testing positive.

4.6 Conclusion

An Ap and Tp of 7.4% and 3.8%, respectively was recorded in this study and no statistically significant production losses were identified. The majority of study farms engaged in high risk management practices for JD transmission. Although the average dairy herd size in Ireland is relatively small, which may be protective against MAP transmission, the abolition of EU milk quotas is leading to dramatic increases in herd size in Ireland. As this study reiterates the increased risk of JD in larger herds it will be necessary to repeat economic studies to monitor the impact of changing demographics in national herds.
Chapter 5: Results of a five year Johne's disease control programme
5.1 Abstract

Johne's disease (JD) continues to present diagnostic difficulties, thereby hampering on-farm control programmes. A combined approach of improved calf management and selective culling currently forms the basis of many JD control programmes. A longitudinal study has never been conducted in an Irish context, to investigate if JD sero-prevalence could be reduced following control programme implementation, and therefore was our objective. A secondary objective was to investigate if repeatedly elevated ELISA results could be used as a predictor of JD pathological changes.

A 139 cow dairy herd was recruited to the study. An intensive ELISA testing regime was implemented initially on an almost monthly basis, and was subsequently reduced to quarterly sampling. On-farm management changes were implemented on the basis of ELISA results e.g. ELISA positive cows calved in isolation. Two selection criteria were used to identify cows for post-mortem (PM) examination; i) multiple ELISA seropositive results and confirmed positive by faecal culture, and ii) multiple ELISA seropositive results but not confirmed positive by faecal culture. Logistic regression was used to identify associations between PM findings indicative of JD and ELISA S/P ratio sub-categories (≥140, ≥150, ≥180, and ≥200), number of ELISA tests, parity and breed.

In the initial herd test 7.9% tested MAP ELISA positive (2012), with 1.4% positive in the final herd test (2016). In total 15 animals underwent PM, with three displaying JD signs at PM. No significant association was identified between ELISA S/P ratio and PM signs.

Implementation of management changes led to a successful reduction in herd prevalence of ELISA positive cows, long term commitment however is necessary to control JD. Furthermore, ELISA test results were not a definitive indicator of cows with pathological lesions.
5.2 Introduction

Control of Johne’s disease (JD) within a herd presents a considerable challenge to livestock farmers and their service providers (Garcia and Shalloo, 2015). *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the etiological agent of JD, is a slow growing bacterium and infection can remain latent in infected animals for many years, making diagnosis difficult (Nielsen and Toft, 2008). Although culture is considered the gold standard ante mortem test for MAP detection, ELISA testing is favoured for herd screening as a more practical and lower cost diagnostic tool (Böttcher and Gangl, 2004). ELISA is also considered predictive for an increased likelihood of MAP faecal shedding (Collins, 2002). All test methodologies available for MAP (culture, PCR, ELISA), are limited in terms of sensitivity (Se) and specificity (Sp), although advanced stages of JD are associated with improved diagnostic accuracy (Gilardoni et al., 2012).

Clinical manifestations of advanced JD include progressive diarrhoea and weight loss (Garcia and Shalloo, 2015), resulting from thickening and corrugation of the intestinal mucosa (Buergelt et al., 1978). A desire to address these animal health and welfare issues in cattle has stimulated interest in JD control, particularly in dairy herds. The economic losses associated with MAP infection at farm level have also driven interest in controlling the disease internationally (Geraghty et al., 2014). The prevalence of MAP sero-positivity in Irish herds compares favourably with other dairying nations (Good et al., 2009, Nielsen and Toft, 2009), however, and to date in the Republic of Ireland (Ireland), no association has been identified between subclinical MAP infection and dairy cow production parameters (Hoogendam et al., 2009, Kennedy et al., 2016). A final concern driving control programme implementation is the speculated zoonotic link between MAP and Crohn’s disease in humans (Feller et al., 2007). Ireland is heavily reliant on dairy exports and infant formulae markets however (DAFM, 2014), which is, perhaps, the most compelling motive for Irish farmers to engage in JD control. Should the postulated zoonotic link between MAP and Crohn’s disease in humans be proven, it is estimated that it will cost the dairy industry internationally billions of dollars (Groenendaal and
Zagmutt, 2008). Consequently, the potential zoonotic risk alone makes it incumbent on farmers globally to employ measures to limit the levels of MAP in milk destined for the human food chain.

Simulation studies have identified improved calving and calf management as the most important element of JD control on farm (Groenendaal et al., 2002). Other studies have investigated using a ‘test and cull’ policy (Lu et al., 2008), the overall focus being to break the cycle of disease transmission from older to younger animals. A combined approach of improved calf management and selective culling, therefore, currently forms the basis of many JD control programmes (Nielsen, 2009b, Collins et al., 2010). Once identification of potentially infected cows is achieved, implementation of herd management changes is facilitated. Informed decisions can be made with regard to colostrum and feed management of young calves, manure management, and annual culling decisions.

Internationally longitudinal studies have been conducted to monitor the success of various JD programmes (Pillars et al., 2009, Collins et al., 2010). During a six year field trial, Collins et al. (2010), achieved a 6% reduction in ELISA sero-prevalence by (i) employing ELISA testing to identify high risk animals, (ii) managing the calving area and colostrum feeding based on test status of each cow, (iii) employing measures to limit calf contact with faeces e.g. 'calf-snatching at birth' and, (iv) selective culling of repeatedly seropositive cows. A longitudinal study has never been conducted in an Irish context, however, to investigate if JD sero-prevalence could be reduced following control programme implementation. Ireland initiated a voluntary pilot JD control programme coordinated by Animal Health Ireland (AHI) in 2014. Given this fact and the differences between Ireland and other jurisdictions in terms of MAP prevalence and impact, a longitudinal study would prove useful in investigating the likely success to be expected from a national JD control programme. Our objective, therefore, was to conduct an observational study on an Irish dairy farm using a comprehensive system of continuous herd monitoring facilitating introduction of targeted management changes. A secondary objective of our study was to
investigate if repeatedly elevated ELISA results could be used as a predictor of pathological changes in the gastrointestinal tract of high risk cows.

5.3 Materials and methods

5.3.1 Study Herd

A 139 cow pasture-based dairy herd was recruited to the study in 2012. The farm was located in the dairy dense province of Munster in the Republic of Ireland (Figure 5.1). The farm was 48 hectares in size, and had a soil type of free-draining acid brown earth of sandy loam to loam texture, divided into 96 paddocks to facilitate rotational grazing. The herd was entirely spring-calving and cows were typically turned out to pasture directly post-calving (January to April) to achieve a 300-day grazing season. All male calves were sold at two weeks of age. Heifer calves were transferred to an off-site heifer rearing facility post-weaning, and returned to the adult herd as in-calf heifers approximately two months prior to calving.

This dairy farm was depopulated in 1997, as per statutory requirement, following a confirmed case of bovine spongiform encephalopathy (BSE). Herd repopulation was completed in 1998. A strict biosecurity protocol was implemented when purchasing heifers post-depopulation. This included a strict testing regime for all animals purchased (O Farrell et al., 2001). The animals under investigation in the current study, therefore, largely consisted of descendants of repopulation cows. Over the course of this study however, animals from 8 different herds of origin were identified.
The predominant breed on the farm between 2012 and 2016 was Holstein-Friesian (HF), the remainder being Jersey or Jersey crossbreeds (JEX). Prior to commencement of the study, farm health records post-1998 were examined to identify any previously diagnosed and/or confirmed clinical cases of JD in the herd. The study was conducted between May 2012 (Year 1 (Y1)) and July 2016 (Year 5 (Y5)). The first year of the study (Y1) was used to establish initial within-herd prevalence of ELISA positives and complete a parallel study reported by Kennedy et al. (2014a). In Y2, Y3, Y4, and Y5 management changes were
implemented based on Y1 ELISA results. The herd entered the voluntary AHI pilot JD control programme in 2014.

5.3.2 Sample Protocol

Sampling was conducted under license from the appropriate authority in each year of the study (years 1 and 2 were licensed by the Irish Department of Health and Children; years 3, 4, and 5 were licensed by the Health Products Regulatory Authority, license number (AE19132/P027). The ELISA, PCR and faecal culture testing regimens applied throughout the course of the study are outlined in Table 5.1. Briefly, blood sampling was initially conducted as close to monthly as logistics would allow. This was subsequently reduced to quarterly sampling as herd status became more clearly defined. Faecal PCR and culture were employed at various time points throughout the course of the study to confirm ELISA results.

As required for the Irish bovine tuberculosis (bTB) eradication programme, the study herd was tested annually for bTB using the single intradermal cervical comparative test (SICCT). SICCT has been shown to interfere with ELISA readings for up to 71 days post-bTB testing (Kennedy et al., 2014a). A proportion of samples collected in Y1 to Y3, therefore, were collected within this period of interference. As ELISA testing in subsequent years was completed at lengthier test intervals, interference from SICCT was avoided.
Table 5.1: Sampling regime across each year of the study.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Individual</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blood ELISA samples</td>
<td>Monthly* (May to</td>
<td>Monthly* (February,</td>
<td>Monthly* (January to</td>
<td>Quarterly: (January,</td>
<td>January and July</td>
</tr>
<tr>
<td></td>
<td>November)</td>
<td>May to November)</td>
<td>Dec)</td>
<td>May, August and November</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Individual</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>milk ELISA samples</td>
<td>Monthly (May-</td>
<td>Monthly (May-</td>
<td>Not conducted</td>
<td>Not conducted</td>
<td>Not conducted</td>
</tr>
<tr>
<td></td>
<td>November)</td>
<td>November)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Faecal Sampling</strong></td>
<td>All ELISA positive cows (PCR and culture)</td>
<td>All ELISA positive cows (culture)</td>
<td>All ELISA positive cows (culture)</td>
<td>All ELISA positive cows (culture)</td>
<td>All ELISA positive cows (culture)</td>
</tr>
</tbody>
</table>

Y= year

*For the purposes of an additional study, sampling frequency increased in the post-TB test period (Kennedy et al 2014)

5.3.3 Sample preparation and analysis

Blood samples were collected in tubes that did not contain anticoagulant. Samples were centrifuged for five minutes at 4000g and serum collected into 1.5 mL microtubes for analysis and storage. Milk samples were transferred into 1.5 mL microtubes and defatted by centrifuging at 20,000g for one minute. Skim milk was then transferred to clean 1.5mL microtubes for analysis and storage at -20°C. Both serum and milk samples were tested by a commercial laboratory ISO17025 accredited for MAP ELISA testing (FBA Laboratories, Cappoquin, Co. Waterford, Ireland). This laboratory is also designated by AHI for the Irish voluntary JD control programme (www.animalhealthireland.ie).

All samples (serum and milk) were tested using the ID Screen Paratuberculosis Indirect Screening Test (ID Vet, Montpellier, France). This test is an *M. phlei* absorbed ELISA which detects anti-MAP IgG. Results were reported as a sample to positive ratio (S/P ratio). The test has a reported sensitivity (Se) in serum of 41.5 % and specificity (Sp) of 99.4% (Fry et al., 2008). Results were reported as sample to positive ratio (S/P ratio) calculated using the formula S/P...
ratio = \frac{((\text{OD Sample} - \text{OD Negative control}) \times 100)}{\text{OD Positive control} - \text{OD Negative control}}. \quad \text{For the purposes of reporting within-herd MAP prevalence, samples were categorised as positive or negative according to manufacture instructions i.e. \geq 70 \text{ S/P classified as seropositive for serum. Further sub-categories of 'high' seropositive animals were established using S/P ratio cut-offs of \geq 140, \geq 150, \geq 180, and \geq 200 for the purposes of logistic regression.}

Faecal samples collected from ELISA positive cows in Y1 were tested using real-time PCR (rtRT-PCR) and faecal culture using 'in-house' methodologies developed by Cork Institute of Technology (Douarre et al., 2010). The target gene for PCR was IS900 and the primer sequences used for DNA amplification were \text{5'}-\text{GAAGGGTGTTCGGGGCCGTCGCTTAGG-3'} and \text{5'}-\text{GGCGTTGAGGTCGATCGCCCACGTGAC-3'}. Faecal samples collected in Y2 to Y5 were submitted to the Irish Central Veterinary Research Laboratory (Backweston, Cellbridge, Co. Kildare) as required by the Irish JD pilot programme. Faecal samples were cultured using the automated TREK ESP® para-JEM® system (Thermo Scientific). Confirmation was performed using Herrolds Egg Yolk agar slopes both with and without mycobactin. Faecal culture was performed either as described by Britton et al, (2017). A faeces sample weighing 2g was placed in 35 mL sterile distilled water. This solution was then manually shaken and allowed to stand for 30 minutes. Subsequently, 5 mL was removed from the top of the solution using a Pasteur pipette and placed into 25 mL 0.9% hexadecylpyridinium chloride (HPC) in 50% brain heart infusion broth (BHIB) for overnight incubation at 37 °C.

The next day, faecal samples were centrifuged at 3000g for 20 minutes at 15 °C. The supernatant was discarded and 1ml of an antibiotic mix from the para-JEM® Antibiotic Supplement in 50% BHIB was added to the pellet and allowed to incubate at 37 °C overnight. The following day, para-JEM® bottles were inoculated with 1 mL para-JEM® Growth Supplement, 1 mL para-JEM® Egg Yolk Supplement, 0.5 mL para-JEM® Antibiotic Supplement and 0.05 mL para-
JEM® BLUE before the addition of 0.5 mL of each sample. The para-JEM® bottles were vortexed for 2 minutes, the top of the bottles were swabbed with 70% alcohol and the TREK connectors were attached, prior to being placed in the TREK ESP® culture system at 37 °C for a minimum of 42 days (Britton et al., 2017).

The para-JEM® bottles were removed from the automated system and shaken for 2 minutes and the TREK connector removed, before the contents were stained using the Ziel Nielsen method to identify acid fast bodies (AFB). Samples positive for AFB were subjected to PCR confirmation and cultured on Herrolds Egg Yolk agar slopes both with and without mycobactin and incubated at 37 °C for a minimum of 8 weeks (Britton et al., 2017).

5.3.4 Management Protocols

Peer-reviewed literature was explored to identify best practice management recommendations for limiting the transmission of MAP on farm (Nielsen et al., 2007, Cashman et al., 2008, Nielsen and Toft, 2011, Dörë et al., 2012, Sweeney et al., 2012). This investigation was used to compile an appropriate and practical JD management programme for the farm to be implemented from Y2 of the study. Colour-coding of cows based on a version of the Danish risk identification system (i.e. red cow = high risk, green cow = low risk) (Nielsen et al., 2007) was implemented on the study farm on the basis of Y1 and Y2 ELISA results. A cow recording at least one ELISA positive result was deemed high risk and categorised as a ‘red cow’. All cows recording only ELISA negative results were deemed lower risk and categorised as ‘green’. From the 2014 calving season onwards red cows were calved in isolation and their calf removed to an individual calf pen immediately after birth. The dam was returned to the main milking herd within 24 hours of calving. Replacement heifer calves from red cows were tagged at birth for ease of identification within the herd over the duration of the study. Colostrum from red cows was discarded and their calves were fed colostrum from a consistently ELISA negative dam. All other calves received colostrum from either their own dam or
a test negative donor, as required. The practice of pooling colostrum was discontinued. Cleaning and re-bedding of the calving area with clean straw between each calving was targeted, although practically not achievable in all cases due to housing capacity and the high volume of cows calving in this spring-calving system. Additionally, any calf born outside of the dedicated calving area, e.g. born prior to expected calving date in the dry cow area, was assigned red status, as these calves were potentially exposed to faecal material from a number of cows. If female, these calves were not retained as replacement heifers. The use of milk replacer was strongly advocated for continued calf feeding but could not practically be introduced until year 3 of the study. High somatic cell count and antibiotic milk was not used for feeding replacement heifer calves. To maximise compliance, management changes were examined for practicality on the current study farm, and for applicability to typical commercial Irish dairying systems during busy spring-calving periods when labour resources may be limited (Gleeson et al., 2008).

5.3.5 Selection criteria for clinical and post-mortem examination (PM)

Cows seropositive for MAP i.e. recording ≥ one ELISA positive result (Table 5.2), were subject to regular veterinary clinical examinations by research veterinarians based at Teagasc, Moorepark, Co. Cork, Ireland. A total of two selection criteria were used to identify cows for PM examination which included; i) cows recording multiple ELISA seropositive results and confirmed positive by faecal culture, and ii) cows due for routine end of lactation culling which recorded multiple ELISA seropositive results but were not confirmed positive by faecal culture. In addition, a single cow due for routine end of lactation culling but having a shared source-herd ancestry with a faecal culture positive cow was also subject to necropsy.

All cows were euthanized by lethal injection of pentobarbital, administered intravenously by the veterinary practitioner with a duty of care to animals on the farm. Necropsies were conducted by the Department of Agriculture, Food and
the Marine's (DAFM) Veterinary Laboratory Service, Co. Cork, Ireland (11 cows) and University College Dublin's (UCD) School of Veterinary Medicine, Dublin, Ireland (4 cows). Regardless of location, PM sampling was conducted using a standard protocol. A comprehensive gross examination of each cow was conducted. Tissues taken for histopathological examination were fixed in 10% formalin immediately on sampling. Tissues included proximal, middle and distal jejunum, ileum, ileocaecal valve and lymph node, proximal and distal caecum, and multiple sections of colon and mesenteric lymph nodes. Tissue smears were taken for direct Ziel Nielsen staining (Britton 2017). Photographic images were taken of abnormal lesions.

5.3.6 Data and Statistical Analysis

Descriptive analysis, dataset construction, and graphical representations were completed in Excel (MSOffice2010). All further statistical analyses including Pearson correlation and logistic regression were completed in Stata Version 12 (StataCORP, USA).

The within-herd apparent prevalence at each test date of the study was calculated as the number of ELISA positive animals divided by the total number of animals tested. Apparent prevalence was calculated for both milk and blood samples. Box plots were constructed to display trends in serum ELISA S/P ratios across the duration of the entire study. Logistic regression was used to identify any association between PM findings indicative of JD (dependent variable) and ‘high’ positive S/P ratio sub-categories (≥140, ≥150, ≥180, and ≥200), number of ELISA tests, parity (1 to 9) and breed (HF, JEX) (independent variables). As a first step, a univariable analysis was performed for each independent variable. Multivariable logistic regression was subsequently conducted using a manual backwards elimination with a forward step. A significance level of $P<0.05$ was chosen for retention of independent variables in the final model. Number of tests conducted was forced into the model when examining associations between PM signs and S/P ratio sub categories. Due to the issue of quasicomplete-separation for completing regression analysis on PM findings and breed, we used a penalized likelihood logistic regression model
using the penlogit command in stata. We set priors of 0 and 2.5 to yield a conservative estimate of odds ratio by using a heavy tailed Cauchy prior distribution (Gelman et al., 2008, Rainey, 2016).

5.3.7 Testing costs

Quotes were obtained from Irish accredited laboratories to facilitate estimation of costs associated with implementing the testing regime adopted in the current study. An initial quote was obtained based on a standard commercial price per test (€4). A volume discount quote was also requested based on a minimum number of tests to be conducted over a specified time period. Testing costs over the current study were calculated based on the number of serum ELISA tests alone, conducted in the non-SICCT influenced period. Faecal culture and PCR testing costs were not included in the overall calculation.

5.4 Results

5.4.1 Herd clinical history

From herd repopulation in 1998 to commencement of this study, no clinical case of JD had been diagnosed on the study farm. Additionally no cow displayed clinical signs consistent with JD throughout the five years of the current study, with a single exception. Following faecal culture confirmation of Cow 1 as JD infected, she was immediately dried off at a period of peak lactation and placed in isolation. Within seven days, she had developed diarrhoea and began losing body condition. As Cow 1 never previously displayed clinical signs of JD, it is likely that the stress associated with isolation and drying off resulted in clinical signs emerging in this animal.

5.4.2 Year-by-year herd results

ELISA, faecal PCR, faecal culture and PM
A graphical representation of within-herd seroprevalence and PM time points across the five years of study is included in Figure 5.2. The trends in serum ELISA S/P ratios across the five years are shown in Figure 5.3. Across the duration of the study, 30 animals recorded at least one seropositive ELISA result (Table 5.2). Following the removal of a confirmed JD positive cow in 2013, an overall reduction in the herd sero positivity was noted. This included seven cows that generated positive antibody responses reverting back to seronegative status and remaining negative for their duration in the study. A year-by-year outline of herd test results, including pathological examinations, is included in the following paragraphs.
**Figure 5.2:** Herd prevalence of serum ELISA positives across the study excluding SICCT influenced period.
Figure 5.3: Trends in serum S/P\% ratio across each study year.

SP ratio capped at 300.
Table 5.2: Results of cows that recorded a minimum of one serum ELISA positive result over the study. Cows selected for PM are also identified.

<table>
<thead>
<tr>
<th>Cow</th>
<th>Breed</th>
<th>Born</th>
<th>PCR</th>
<th>Culture Pos.</th>
<th>PM</th>
<th>Outcome</th>
<th>Number of ELISA positive tests recorded in each study year*</th>
<th>Total Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 J</td>
<td>J</td>
<td>2003</td>
<td>++</td>
<td>2013</td>
<td>2013</td>
<td>JD+</td>
<td>5(5) 3(3)</td>
<td>8</td>
</tr>
<tr>
<td>2 J</td>
<td>J</td>
<td>2009</td>
<td>+</td>
<td>2013</td>
<td>PM</td>
<td>NAD</td>
<td>5(5)</td>
<td>5</td>
</tr>
<tr>
<td>3 F</td>
<td>F</td>
<td>2009</td>
<td>+</td>
<td>2013</td>
<td>PM</td>
<td>NAD</td>
<td>5(5) 5(5)</td>
<td>10</td>
</tr>
<tr>
<td>4 F</td>
<td>F</td>
<td>2009</td>
<td>+</td>
<td>2013</td>
<td>PM</td>
<td>NAD</td>
<td>5(5) 5(5)</td>
<td>10</td>
</tr>
<tr>
<td>5 F</td>
<td>F</td>
<td>2009</td>
<td>++</td>
<td>2013</td>
<td>PM</td>
<td>NAD</td>
<td>5(5) 5(5)</td>
<td>10</td>
</tr>
<tr>
<td>6 J</td>
<td>J</td>
<td>2010</td>
<td>x</td>
<td>2013</td>
<td>PM</td>
<td>NAD</td>
<td>5(5) 0(5)</td>
<td>10</td>
</tr>
<tr>
<td>7 F</td>
<td>F</td>
<td>2009</td>
<td>++</td>
<td>2013</td>
<td>PM</td>
<td>NAD</td>
<td>4(5) 2(5)</td>
<td>10</td>
</tr>
<tr>
<td>8 F</td>
<td>F</td>
<td>2009</td>
<td>+</td>
<td>2013</td>
<td>PM</td>
<td>NAD</td>
<td>4(5) 3(5)</td>
<td>10</td>
</tr>
<tr>
<td>9 F</td>
<td>F</td>
<td>2011</td>
<td></td>
<td></td>
<td>2013</td>
<td>PM NAD</td>
<td>5(5)</td>
<td>5</td>
</tr>
<tr>
<td>10 F</td>
<td>F</td>
<td>2011</td>
<td></td>
<td></td>
<td>2013</td>
<td>PM NAD</td>
<td>1(5)</td>
<td>5</td>
</tr>
<tr>
<td>11 F</td>
<td>F</td>
<td>2011</td>
<td></td>
<td></td>
<td>2013</td>
<td>PM NAD</td>
<td>5(5)</td>
<td>5</td>
</tr>
<tr>
<td>12 J</td>
<td>J</td>
<td>2007</td>
<td></td>
<td></td>
<td>2013</td>
<td>PM NAD</td>
<td>0(5) 0(5)</td>
<td>10</td>
</tr>
<tr>
<td>13 J</td>
<td>J</td>
<td>2006</td>
<td>2015</td>
<td>2015</td>
<td>JD+</td>
<td></td>
<td>0(5) 0(5) 2(8) 3(4)</td>
<td>22</td>
</tr>
<tr>
<td>14 J</td>
<td>J</td>
<td>2011</td>
<td>2015</td>
<td>2016</td>
<td>JD+</td>
<td></td>
<td>0(5) 0(8) 2(4) 1(1)</td>
<td>18</td>
</tr>
<tr>
<td>15 J</td>
<td>J</td>
<td>2011</td>
<td>2015</td>
<td>2016</td>
<td>PM</td>
<td>NAD</td>
<td>0(5) 0(8) 1(4) 0(1)</td>
<td>18</td>
</tr>
<tr>
<td>16 F</td>
<td>F</td>
<td>2006</td>
<td>x</td>
<td>Exit Nov 2015</td>
<td>2(5)</td>
<td></td>
<td>1(5) 1(8) 0(4)</td>
<td>22</td>
</tr>
<tr>
<td>17 J</td>
<td>J</td>
<td>2007</td>
<td></td>
<td>Exit Dec 2014</td>
<td>0(5)</td>
<td></td>
<td>0(5) 1(4)</td>
<td>14</td>
</tr>
<tr>
<td>18 J</td>
<td>J</td>
<td>2010</td>
<td></td>
<td>Exit Oct 2015</td>
<td>0(5)</td>
<td></td>
<td>1(5) 0(8) 0(3)</td>
<td>21</td>
</tr>
<tr>
<td>19 F</td>
<td>F</td>
<td>2010</td>
<td></td>
<td>Present</td>
<td>0(5)</td>
<td></td>
<td>0(5) 1(8) 0(4) 0(2)</td>
<td>24</td>
</tr>
<tr>
<td>20 F</td>
<td>F</td>
<td>2011</td>
<td></td>
<td>Exit Nov 2015</td>
<td>0(5)</td>
<td></td>
<td>0(5) 1(8) 0(4)</td>
<td>17</td>
</tr>
<tr>
<td>21 F</td>
<td>F</td>
<td>2011</td>
<td></td>
<td>Present</td>
<td>0(5)</td>
<td></td>
<td>1(8) 0(4) 0(2)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>J</td>
<td>2011</td>
<td>Exit Nov 2015</td>
<td>1(5)</td>
<td>0(8)</td>
<td>0(4)</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>2007</td>
<td>Exit Nov 2012</td>
<td>5(5)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>2007</td>
<td>Exit Jan 2013</td>
<td>1(5)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>J</td>
<td>2009</td>
<td>Exit Nov 2012</td>
<td>5(5)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>F</td>
<td>2010</td>
<td>Present</td>
<td>1(1)</td>
<td>0(2)</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>F</td>
<td>2012</td>
<td>Present</td>
<td>0(8)</td>
<td>2(4)</td>
<td>1(2)</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>J</td>
<td>2012</td>
<td>Present</td>
<td>0(8)</td>
<td>1(4)</td>
<td>2(2)</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>F</td>
<td>2009</td>
<td>Present</td>
<td>0(5)</td>
<td>0(5)</td>
<td>0(8)</td>
<td>0(4)</td>
<td>1(2)</td>
</tr>
</tbody>
</table>

PM: Post Mortem

Number of ELISA positive tests recorded in each study year*: only test conducted under non-SICCT influenced period included. 2014*: From March 2014 to May 2015 the entire herd tested ELISA negative.

PCR: + = Weak positive, ++= Strong positive, x= negative. The remainder of animals were either ELISA negative or not present in the herd at this time (Year 1) and did not have a sample tested for PCR.

Outcome: NAD: No abnormalities detected at PM, JD+: confirmed JD at PM or by histopathology. Cow 16-22 (& cow13) recorded ELISA positive results pre March 2014
A total of 11 cows (7.9%) tested MAP seropositive at the initial herd test (May 2012). A lower number of positive individuals (6%) were identified by milk ELISA. With the exception of the post-SICCT period of interference, monthly serum ELISA results in 2012 remained relatively static (range 7.2%- 9.3%) with the same cows generally remaining seropositive (Table 5.2 and Figure 5.4). The highest herd seroprevalence was recorded in November 2012 i.e. late lactation. Milk ELISA also recorded an increase in within-herd prevalence in November 2012, although to a much greater degree (14.4%). Regarding faecal testing, 10 seropositive cows recorded at least one positive faecal PCR result (Table 5.2). The number of PCR positives may be as a result of the “pass through” phenomenon (Behr et al., 2010). Faecal culture did not yield any positive results. The study herd had an annual culling rate of 25% and as part of this annual end of lactation cull, a number of seropositive cows (Table 5.2: Cows 23-26) were removed from the herd for non-JD related reasons such as age, not in calf and high somatic cell count.

**Figure 5.4:** Trends in serum S/P ratios of cows recording seropositive results (Cow 1-30)
In February 2013, 7% of the herd tested seropositive. PCR was not continued into 2013, but a single cow recorded a faecal culture positive result in May 2013 (Cow 1). Cow 1 was the first cow selected for isolation and PM based on generating consistently positive ELISA results. Of the cows selected for PM across the entire study, Cow 1 recorded the highest ELISA S/P ratio (Table 5.3). On three occasions in 2012, however, her milk ELISA results classified her as negative. She was a nine year old, seventh lactation, JEX cow, had calved down and been successfully re-bred every year of her lactating life following purchase as an in-calf heifer. As previously mentioned, following a faecal culture positive result, Cow 1, was immediately dried-off, isolated from herd cohorts in an indoor pen, fed a hay-based diet, and only then began to display clinical signs of JD. During the two week period post-isolation, the cow developed intractable diarrhoea and began losing body condition (from BCS 3.5 to BCS 3.0), although remaining bright, alert and responsive. She was subsequently euthanized and subjected to full PM.

PM revealed severe thickening of the jejunum and ileum (Figure 5.5). Mesenteric lymph nodes were enlarged. Acid fast bacilli were identified on Ziel Nielsen smears of the distal ileal mucosa. There was also enlargement of hepatic and mammary lymph nodes. Thickening of bile ducts was reported. On histology there was diffuse, severe stunting, blunting and fusion of both illeal and jejunal villi. The mucosa and submucosa of the jejunum and ileum were severely thickened with a diffuse granulomatous infiltrate, with scattered multinucleated giant cells. Histological examination of lymph nodes identified a sheet-like granulomatous infiltrate that extended through the cortex and medulla, leading to disruption of the normal architecture. A morphological diagnosis of chronic, severe, diffuse, granulomatous enteritis with associated severe lymphadenopathy was made, consistent with an aetiological diagnosis of JD (Figure 5.5).
**Figure 5.5:** Cow 1 displayed classical signs of JD on both gross and histopathology.

**Table 5.3:** Highest and lowest serum ELISA S/P results recorded by PM cows. Non-SICCT influenced tests only. Culture positive and PM positive cows are identified.

<table>
<thead>
<tr>
<th>Cow ID</th>
<th>Parity</th>
<th>BREED</th>
<th>Highest S/P</th>
<th>Lowest S/P</th>
<th>PM signs</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>J</td>
<td>382</td>
<td>86.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>J</td>
<td>193</td>
<td>120.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>F</td>
<td>191</td>
<td>87.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>F</td>
<td>184</td>
<td>95.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>F</td>
<td>284</td>
<td>121.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>J</td>
<td>154</td>
<td>44.62</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>F</td>
<td>99</td>
<td>31.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>F</td>
<td>110</td>
<td>59.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>F</td>
<td>150</td>
<td>70.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>F</td>
<td>111</td>
<td>51.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>F</td>
<td>162</td>
<td>120.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>J</td>
<td>40</td>
<td>14.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>8</td>
<td>J</td>
<td>254</td>
<td>1.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>J</td>
<td>124</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>J</td>
<td>76</td>
<td>8.1</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

134
Following removal of Cow 1, herd seroprevalence began to gradually decrease. The final serum ELISA herd test in Y2 recorded a prevalence of 4.3%. Similar to Y1, however, an increase in milk ELISA positives was recorded in late (3.7%) compared to early and mid-lactation (1.4%). Based on confirmation of a case of JD, 11 further MAP seropositive cows were added to the annual routine end of lactation culling list. These underwent full PM examination following euthanasia. Although seven of these cows had recorded at least a single PCR positive result in Y1 (Table 5.2), selection for PM was based on ELISA results alone. The highest ELISA S/P ratio recorded by each is included in Table 5.3. None of these cows displayed clinical signs of JD prior to euthanasia. At PM, gross findings were unremarkable. Mild lymphocytic infiltration was noted in the lamina propria of ileal sections of a proportion of these cows. However, no histopathological signs consistent with JD were identified.

Y3: In January 2014, five seropositive cows were identified (Cows 13, 16, 17, 19 and 21), only two of which (Cows 13 and 16) had previously tested positive. By February, only two cows tested seropositive (Cows 13 and 20). From March 2014 to December 2014 all cows tested seronegative (excluding the post-SICCT period), including eight cows that had previously recorded at least one positive result since initiation of the study (Cows 13, 16, 17, 18, 19, 20, 21, 22). No culture positive results were recorded in 2014.

Y4: All cows remained seronegative until May 2015 when two animals tested seropositive (Cows 13 and 28). An additional four cows yielded positive results in 2015. Cows 14, 15, and 29 were seropositive in August, and cow 27 yielded a seropositive result in November. Of these seropositives, three were identified as faecal culture positive (cows 13, 14, and 15), the remainder being faecal culture negative.
In November 2015, prior to reporting of faecal culture results, cow 13, a 9 year old JEX, was selected for culling and PM based on age and MAP seropositivity. She was a purchased cow. Cow 13 was not displaying any clinical signs of JD prior to PM. Gross pathology was unremarkable, with no visible abnormalities observed. Histopathological examination, however, yielded lesions consistent with JD i.e. granulomatous enteritis with giant cell macrophages.

Y5: In January 2016, three cows tested seropositive (cows 14, 28, and 29) all of which had previously tested seropositive in 2015. Cows 14 and 15 which had also recorded faecal culture positive results in December 2015, were selected for PM. Reported days to positivity on faecal culture were 29 and 35 days respectively. Both were five year old JEX cows and were home bred. Cow 14 showed gross and histopathological lesions consistent with JD. Cow 15 displayed no signs of JD on gross pathology or histopathology. Two animals tested seropositive in the final herd test of the current study (July 2016), one of whom had previously tested seropositive (Cow 29). Both cows tested negative on faecal culture. Of cows seropositive which subsequently reverted to ELISA negative status in Y3, two remained in the herd in Y5. Both continued to test seronegative. Animals reared according to newly introduced JD control management practices entered the milking herd in spring of Y5. None recorded a positive ELISA result by study termination.
Table 5.4: Univariable logistic regression. Any variables showing P value <0.1 are highlighted in bold

<table>
<thead>
<tr>
<th></th>
<th>PM Signs</th>
<th>Breed S/P140</th>
<th>S/P150</th>
<th>S/P180</th>
<th>S/P200</th>
<th>Total No. Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed</td>
<td>0.02</td>
<td>0.79</td>
<td>0.83</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>S/P140</td>
<td></td>
<td>0.79</td>
<td>0.83</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>S/P150</td>
<td></td>
<td></td>
<td>0.31</td>
<td>0.83</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>S/P180</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td>S/P200</td>
<td>0.06</td>
<td>0.06</td>
<td>0.45</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Parity</td>
<td>0.15</td>
<td>0.15</td>
<td>0.78</td>
<td>0.78</td>
<td>0.13</td>
<td>0.07</td>
</tr>
<tr>
<td>Culture Pos</td>
<td>0.02</td>
<td>0.02</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
<td>0.11</td>
</tr>
</tbody>
</table>

5.4.3 Statistical Analysis

Results of univariable analysis are shown in Table 5.4. Six PM animals recorded S/P ratios higher than 140 MAP S/P, however no association was identified with PM lesions (p = 0.43) (Table 5.5). Similarly no significant association was identified between recording S/P ratio >200 (3 cows) and PM signs.

5.4.4 Cost of programme

Taking ELISA costs of €4 per cow, testing costs amounted to approximately €13,500. The discounted price of €3 per cow resulted in costs over €10,000. This figure accounts for serum ELISA testing cost alone and does not include milk ELISA tests, sample collection costs or other financial considerations such as cull cow value and cost of replacement stock.
Table 5.5: Logistic regression showing associations between PM signs (dependent variable) and independent variables.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Odds Ratio</th>
<th>P Value</th>
<th>Model (Model P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of PM changes</td>
<td>n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA S/P &gt;140</td>
<td>9</td>
<td>7.5</td>
<td>0.43</td>
</tr>
<tr>
<td>ELISA S/P &gt;150</td>
<td>9</td>
<td>7.5</td>
<td>0.43</td>
</tr>
<tr>
<td>ELISA S/P &gt;180</td>
<td>6</td>
<td>11.7</td>
<td>0.32</td>
</tr>
<tr>
<td>ELISA S/P &gt;200</td>
<td>3</td>
<td>70.2</td>
<td>0.12</td>
</tr>
<tr>
<td>Parity</td>
<td>4.0</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Number of Tests</td>
<td>1.3</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Breed (JEX vs. FRX)</td>
<td>8.19</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

5.5 Discussion

The aim of this longitudinal study was to implement a JD control programme in an Irish dairy herd, and examine if similar progress to that reported internationally could be achieved (Collins et al., 2010, Nielsen and Toft, 2011). We have shown that reductions in within-herd seropositivity can be achieved over a relatively short period of time using a combined approach of culling seropositive cows and optimal calving and calf management. The long term commitment common to MAP control programmes in any jurisdiction, however, is again emphasised. While reductions in seroprevalence were rapidly achievable, we clearly highlight the risk of prematurely declaring successful
elimination of JD from a herd based on serology alone. Our attainment of seronegative herd status was very much transient in nature and again reinforces the necessity of continuous monitoring programmes for JD.

A level of association between ELISA results and JD-associated pathological lesions has previously been reported (Dennis et al., 2008), and culling of repeatedly ELISA positive cows has formed part of successful international JD control programmes (Collins et al., 2010, Nielsen and Toft, 2011). Results from our study, however, support a recent report stating that ELISA results alone, in the absence of clinical signs, should not be used to diagnose infection with MAP (Ramovic, 2016). Additionally, Pinedo et al. (2008), highlights the importance of basing culling decisions on a combination of ELISA and confirmatory testing to enhance the accuracy of identifying MAP infected animals. Our study provides further verification for such an approach, as three of four seropositive and faecal culture positive cows recorded either gross or histopathological signs of JD. Additionally, while our initial culling strategy, based primarily on ELISA results, was successful in reducing within-herd prevalence, it resulted in slaughter of cows that subsequently recorded no pathological signs of JD. Overall, we were unable to identify a significant association between ELISA results and pathological lesions at necropsy over the five years of study. Although absence of pathological changes is not a definitive diagnosis of non-infection, it does seem likely that the ELISA test used in the current study identified a number of false positive animals.

We did expect superior test specificity, in terms of identifying cows with PM lesions, to that ultimately generated, although it should be noted specificity of histopathology is not perfect. Control of JD is generally associated with a cell mediated immune response and antibodies are largely regarded as ineffective against clearance of MAP (Stabel, 2000). Antibody production however, usually equates to disease progression (Stabel, 2000) and development of clinical signs, which would support ELISA specificities of mainly between 90% and 100% as reviewed by Nielsen and Toft (2008). We propose a number of
potential reasons why the specificity of ELISA in the current study might not be as reported internationally (Fry et al., 2008). Firstly, Ireland conducts an extensive bTB testing regimen using SICCT. Kennedy et al. (2014a), highlighted a short-term but extensive impact on MAP sero-prevalence post-SICCT. The possibility of cows becoming sensitised over a more extended period is possible, although not previously reported and requires more in-depth investigation. Secondly, Irish soils contain high levels of environmental mycobacteria (Cooney et al., 1997) and these may result in the production of cross-reacting antibodies, contributing to sub-optimal test specificity. Indeed the reason that the SICCT is conducted in Ireland for detection of bTB is the high likelihood of reactions to environmental mycobacteria (Good and Duignan, 2011).

A final hypothesis we propose is that the presence of a MAP shedder in a herd may result in an anamnestic response in cows previously sensitised to an undefined mycobacterium (which may include MAP), generating a non-specific ELISA result. This is supported by the fact that seven cows that initially generated positive antibody responses in the first two years of the current study, reverted back to seronegative status, following removal of Cow 1 (the index case), and remained seronegative for their remaining lifetime in the herd. However, it must be stated that these cows could subsequently revert to seropositivity as did Cow 13 if followed for a lengthier time period. It may prove worthwhile for MAP ELISA kit manufacturers to provide estimates of test sensitivities and specificities for herds of differing within-herd prevalence of clinical cases, of faecal shedders and of sub-clinical carriers.

It is suggested approximately 10% of infected animals progress to show clinical signs of JD (Sweeney et al., 2012). In the current study with the exception of Cow 1, clinical signs were not evident. Potential reasons include possible increased resilience to MAP infection in the study herd or the prolonged outdoor grazing environment as contact with faeces is minimised compared to full time indoor housing systems. Other potential reasons for low levels of clinical JD
include, expression of the disease being truncated by culling practices (Whittington and Sergeant, 2001). As is common in Ireland, this herd targeted a compact spring calving season aimed at the period of maximum grass growth (Dillon et al., 1995). Breeding is focused on fixed calendar dates in spring and animals found not to be in calf are culled at the end of lactation. This may facilitate removal of subclinical animals that may have been adding to the infectious pressure within a herd. If such stringent culling practices were not in place, prolonged retention of sub-clinically infected cows could eventually result in increased numbers of clinical cases. In an era of dairy herd expansion in Ireland, due to EU milk quota abolition (Shalloo et al., 2012), increasing herd size combined with less strict culling practices may place farmers at increased risks of disease transmission. Education needs to be provided to farmers in the process of farm expansion to negate such risks. Furthermore as two of three animals showing JD signs at PM were purchased animals, the importance of biosecurity and operating a closed herd (Sayers et al., 2013) also needs to be reinforced to farmers.

Previous international studies have examined the association between cow breed and clinical JD. Interestingly in the current study, all faecal culture positive cows, and cows subsequently found to have PM lesions consistent with JD, were JEX. This is in line with previous studies which reported Channel Island breeds as being at higher risk of contracting JD (Withers, 1959, Çetinkaya et al., 1997). While genetic evaluation of serological response to MAP in Irish dairy cows has been evaluated and found to be heritable (Berry et al., 2010), research is required to determine whether Irish dairy cows, particularly FRX breeds, are more resilient to MAP infection compared to cows in other jurisdictions. The rarity of clinical reports of JD in Irish herds may also relate to their extensive exposure to environmental mycobacteria which may assist in controlling infection. As reported by Click (2011), a subspecies of Dietzia, previously classified as Mycobacterium gardonae, is capable of limiting signs of JD in cattle. Whether it is possible for additional mycobacteria to mediate such an outcome warrants further investigation, particularly in an Irish context.
The costs associated with implementation of JD control programmes can be considerable (More et al., 2015). How aggressively a farmer opts to pursue a JD control programme will depend on a number of factors including initial herd prevalence, and whether sale of stock forms an important part of farm income (Sweeney et al., 2012). With regard to our study herd, we chose to follow an intense testing regime in years 1 and 2 to facilitate rapid identification of cows potentially transmitting the disease. Sweeney et al. (2012), stated that the costs incurred are believed to be inversely proportional to the speed of disease eradication and the initial rapid success that we achieved in eliminating herd seropositivity would support this. While ultimately our testing regime contributed to rapid elucidation of herd status, monthly testing would be financially and logistically impractical in a commercial herd. The re-emergence of seropositive, and subsequently faecal positive cows, after 15 months of testing seronegative, also, would do little to motivate a livestock farmer. Fluctuating herd status is likely to lead to disenchantment with testing regimes and to a discontinuance of labour intensive management practices. As has occurred internationally, it may be necessary that the “hidden benefits” of implementing a JD control programme, such as improvements in calf and general herd health (Sorge et al., 2010) need to be highlighted in order for Irish farmers to remain engaged in a programme.

Due to cost, the programme implemented in the current study is unlikely to ever be replicated in a commercial setting. In terms of programme design that would be commercially applicable and would have produced an identical outcome to the eventual results of the study, a regime involving an annual herd test would have successfully identified Cow 1. As evidenced in the final stages of the study however, such a sampling regimen may not have guaranteed successful identification of all culture positive cows, sampling time point being a major determinant on whether an animal would or would not have been identified. In 2015, August provided the most successful serum ELISA results in terms of identification of faecal culture positive cows, but any other test date
would have missed at least one culture positive cow. This could potentially prolong the overall disease control programme. For this particular herd two serum ELISA tests per year (mid and late lactation) would have been appropriate to identify culture positive cows and would be more cost effective than implementing a monthly testing regime. Although this study relates to a single herd, it does highlight potentially useful testing regimens to aid identification of culture positive cows.

5.6 Conclusion

Although clinical signs were not evident in this herd, the identification of culture positive animals in this Irish dairy herd highlights the necessity of implementing a JD control programme. Consistently elevated ELISA test results were not found to be a definitive indicator of JD post-mortem findings which presents difficulties in proposing a rapid screening method for the disease. The use of ELISA and subsequently faecal culture, herd ancestry, and breed, however, will lead to greater confidence in selecting culls.
Chapter 6: The single intradermal cervical comparative test interferes with Johne’s disease ELISA diagnostics

Published in Frontiers in Immunology (2014) Volume 5 | Article 564 |
http://dx.doi.org/10.3389/fimmu.2014.00564
6.1 Abstract

Enzyme-linked immunosorbent assays (ELISA) of milk and serum samples are a routinely used method of screening herds for *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Infection with MAP causes a granulomatous enteritis of ruminants known as Johne's disease (JD). The sensitivity (Se) and specificity (Sp) of MAP ELISAs leads to difficulties in the identification of both infected and infectious animals. Interference with MAP ELISA Se and Sp has been reported in MAP seronegative cows following administration of purified protein derivative (PPD) as part of intradermal testing for bovine tuberculosis (bTB). The aim of this study is to examine the impact of the single intradermal cervical comparative test (SICCT) for bTB, on both serum and milk MAP ELISA tests, in a herd containing both seropositive and seronegative cows pre- SICCT. A secondary objective is to provide appropriate timing of JD ELISA tests in relation to the SICCT.

A herd of 139 cows were serum and milk sampled pre and post SICCT administration. Prior to SICCT, 6% of the herd tested seropositive for MAP using milk ELISA, with 8% positive on serum. ID Screen Paratuberculosis Indirect Screening Test (ID Vet) was used to screen the herd. Within 14 days of PPD administration, a significant increase in the prevalence of seropositive cows was recorded. Identical prevalences were recorded with both test matrices (39%). ELISA values remained significantly higher until day 43 post-SICCT in milk (P=0.850), and day 71 in serum (P=0.602). If the 'new' positives detected post-bTB testing are deemed false positives due to generation of cross-reacting antibodies by administration of PPD, milk would appear a more suitable sample for JD ELISA testing within two months of SICCT.

In summary, sampling for JD utilising milk ELISA should be avoided in the 43 day period following PPD administration, with serum ELISA sampling avoided for an additional 28 days.
6.2 Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (MAP), a member of the Mycobacteriaceae family, causes a chronic granulomatous enteritis known as Johne’s disease (JD) (Ayele et al., 2001). Clinical JD is characterised by diarrhoea and progressive cachexia, which ultimately results in death (Clarke, 1997). Uncertainty exists regarding a potential causal link between MAP and Crohn’s disease in humans (Chamberlin et al., 2001, Feller et al., 2007). The potential damage to the global dairy industry should a link between Crohn’s and MAP be fully substantiated (Groenendaal and Zagmutt, 2008), combined with animal health issues, has prompted the establishment of JD control programmes in a number of countries (McKenna et al., 2006, Kennedy, 2011, Nielsen, 2011). Use of enzyme linked immunosorbent assays (ELISA) to identify animals at risk of being infected with MAP is common in control programmes internationally (McKenna et al., 2006, Nielsen et al., 2007), including Ireland (www.animalhealthireland.ie). ELISA is favoured as a screening test due to its relatively low cost compared to faecal culture or polymerase chain reaction (PCR) (Gilardoni et al., 2012). ELISAs also provide timely results compared to culture methods (Gilardoni et al., 2012). The sensitivity (Se) and specificity (Sp) of MAP ELISAs, however, leads to difficulties in the identification of both infected and infectious individuals (Nielsen and Toft, 2008).

*M. bovis*, the causative agent of bovine tuberculosis (bTB), is an additional pathogenic and definitively zoonotic (Moda et al., 1996) member of the Mycobacteriaceae. To reduce the zoonotic risk posed by bTB, address public/animal health concerns, and limit trade restrictions, a compulsory national eradication programme for bTB was established in Ireland in 1962 (Good, 2006). This eradication programme involves ante-mortem testing of all registered bovines annually using the single intradermal cervical comparative test (SICCT) and post-mortem carcass inspection. All SICCT positive animals (reactors) are slaughtered, the herd of origin is restricted, and additional bTB testing is applied to the herd. The comprehensive nature of the testing programme can lead to some animals being tested up to five times in a single
year (Good et al., 2007). The SICCT utilises intradermal introduction of *M. bovis* and *M. avium* subsp. *avium* purified protein derivatives (bPPD and aPPD) at two different sites on the neck to elicit a delayed hypersensitivity response (Monaghan et al., 1994). Comparative measurements at both injection sites, taken 72 hours post PPD administration, are used to assess infection status (Monaghan et al., 1994). Additional ante-mortem testing methods used internationally for detection of bTB include the single intradermal test and the caudal fold test, both less specific than SICCT (de la Rua-Domenech et al., 2006).

Members of the Mycobacteriaceae family share several antigens which can lead to diagnostic difficulties due to antibody cross reaction (Olsen et al., 2001). Varges et al. (2009), has shown interference by both single and comparative intradermal bTB tests on MAP sero diagnostics in bTB negative animals. The primary purpose of this current study was to investigate the impact of SICCT on the prevalence of ELISA positive results (serum and milk) in an Irish herd containing both MAP ELISA seropositive and seronegative animals over a period of six months. Secondary objectives included comparing milk and serum ELISA readings and investigating whether serum samples could be taken at the 72 hour bTB visit without interference from PPD administration.

6.3 Materials and methods

6.3.1 Study Herd

A 139-cow spring-calving dairy herd (mean-calving date February 19th) was recruited to the study. The study was licenced by the Irish Department of Health and Children (licensing authority for experimentation on animals in Ireland at the time of the experiment). This herd was depopulated in 1997 following a confirmed case of bovine spongiform encephalopathy (BSE). The experimental herd, therefore, consisted of descendants of cows used to repopulate the farm in 1998 (O Farrell et al., 2001). Annual statutory bTB test results were sourced since 1998 to provide a bTB history for the herd. Veterinary records were obtained in order to record a JD history for the herd post-repopulation.
Approximately, 60% of the cows were Holstein Friesian (HF), the remaining 40% purebred Jersey (Je) or Je cross-breeds.

6.3.2 Sample Collection

Milk and serum samples were collected 10 and 13 days prior to administration of the routine compulsory annual SICCT herd test (pre-SICCT). The SICCT was administered by the Department of Agriculture, Food and the Marine (DAFM) approved private veterinary practitioner (PVP) responsible for the care of animals on this farm. This is standard practice for the Irish national bTB eradication scheme. Milk and serum samples were collected every 14 days (approximately) for two months post-SICCT. Samples were collected on a monthly basis for the subsequent four months until the composition of the herd changed materially due to end of lactation culling (longitudinal data). A limit of a seven-day interval between serum and milk sampling was applied in order to consider samples as 'matched' (Table 6.1). Milk samples were not available for all cows at every sampling time point and are reflected in a small variation in sample population between milk and serum samples. Additionally, milk samples were not collected in September 2012 due to an un-related health issue on farm. Faecal samples were collected on a weekly basis from consistently ELISA positive cows from 90 days post-SICCT. These cows were also subjected to a veterinary clinical exam.
Table 6.1: Timetable of serum and milk samples and dates of SICCT

<table>
<thead>
<tr>
<th></th>
<th>Serum Sampling Date</th>
<th>Milk Sampling Date</th>
<th>Days post PPD Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre SICCT</td>
<td>May 29</td>
<td>May 31</td>
<td></td>
</tr>
<tr>
<td>SICCT Test Day 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPD administration</td>
<td>June 11</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SICCT Day 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>June 14</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>SICCT Day 2 – Serum sample only</td>
<td>June 14</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Post SICCT Match 1</td>
<td></td>
<td>June 20</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>June 25</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Post SICCT Match 2</td>
<td>July 11</td>
<td>July 11</td>
<td>30</td>
</tr>
<tr>
<td>Post SICCT Match 3</td>
<td>July 24</td>
<td>July 24</td>
<td>43</td>
</tr>
<tr>
<td>Post SICCT Match 4</td>
<td>August 8</td>
<td>August 8</td>
<td>58</td>
</tr>
<tr>
<td>Post SICCT Match 5</td>
<td>August 21</td>
<td>August 21</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>September 5</td>
<td>No sample</td>
<td>99</td>
</tr>
<tr>
<td>Post SICCT Match 6</td>
<td>October 1</td>
<td>October 1</td>
<td>112</td>
</tr>
<tr>
<td>Post SICCT Match 7</td>
<td>November 1</td>
<td>November 1</td>
<td>143</td>
</tr>
</tbody>
</table>

Serum and milk samples were tested using a commercial ISO17025 accredited laboratory (designated laboratory for Irish voluntary JD control programme) using the ID Screen Paratuberculosis Indirect Screening Test (ID Vet, Montpellier, France). The test is an *M. phlei* absorbed ELISA detecting anti-MAP IgG. Status of the sample was evaluated by examining the sample to positive ratio (S/P ratio) calculated using the formula $S/P \text{ Ratio} = \left( \frac{OD_{sample} - OD_{Negative\ control}}{OD_{Positive\ control} - OD_{Negative\ control}} \right) \times 100$. Faecal samples were tested by microbial culture and real-time polymerase chain reaction (rtRT-PCR) using 'in-house' methodologies developed by Cork Institute of Technology as outlined by Douarre et al. (2010). The target gene was IS900. Primer sequences for the amplification were 5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3' and 5'-GGCGTTGAGGTCGATCGCCC ACGTGAC-3' (reverse primer). Faecal culture was performed as follows: One gram of faeces was added to 20 ml of sterile
distilled water. Samples were vortexed for 1 minute and allowed to stand for 30 minutes. Five ml of the supernatant were added to 25 ml of 0.9% hexadecylpyridinium chloride and left undisturbed overnight at room temperature. Tubes were centrifuged at 1700 g for 20 minutes. The supernatant was removed and the pellet was re-suspended in 1 ml of 50 μg/ml amphotericin B. Harolds Egg Yolk Medium agar containing vancomycin, nalidixic acid and amphotericin B at 50 μg/ml were inoculated with 0.2 ml of the suspension and incubated in sealed 25 cm³ tissue culture flasks at 37 °C for 24 weeks.

6.3.3 Data Analysis

Descriptive analysis, dataset construction, and graphical representations were completed in Excel (MS Office 2010). Normality of datasets was examined visually using ladders of power histograms in Stata (version 12). Additional statistical analyses including chi-squared test, t- test, box plot construction, Spearman rank correlation and generalised estimating equations (GEE) were completed using Stata (version 12).

For the purposes of reporting within-herd MAP prevalence, ELISA S/P ratio results were interpreted according to manufacture instructions. Samples recording S/P ratios of ≥70 S/P and ≥ 15 S/P were categorised as positive in serum and milk samples, respectively. Readings classified as inconclusive under manufacturer recommendations (Serum: 60 > S/P <70 S/P) were categorised as negative for the purposes of the present study. The prevalence of positive cows within the herd was plotted versus trial day. Box plots were constructed to highlight trends in ELISA S/P readings pre- and post-SICCT. Longitudinal milk and serum ELISA results were used to create datasets for statistical analysis. ELISA results were recorded as both a categorical variable (positive, negative) and a continuous variable (ELISA S/P readings). Multivariable GEE was used to investigate differences between pre- and post-SICCT categorical and continuous variables (dependent variables).
Independent variables included in the models were sampling time point (pre-SICCT, post-SICCT), breed (Friesian, Jersey), parity (parities 1, 2, 3, ≥4), and date of calving (January, February, March, April). Second level interactions between independent variables were examined and included in the model at \( P \leq 0.05 \). For categorical variable analysis, a binomial distribution was assumed and a logit link function used. For continuous variable analysis, a Gaussian distribution and an identity link function was used. An exchangeable correlation was applied to both analyses. To investigate the correlation between milk and serum ELISA results, Spearman correlation \( (r_s) \) was performed on categorical data sets.

6.4 Results

6.4.1 TB Test

Results of statutory bTB testing for this farm over the past eight years indicate minimal issues with bTB in this herd. Similarly, no bTB positive reactor was identified following SICCT in 2012. From herd repopulation in 1998 to commencement of this study, no clinical case of JD had been diagnosed on the study farm.

6.4.2 MAP ELISA

Prior to administration of the SICCT, a total of 11 of 139 cows (7.9%) tested MAP ELISA positive in serum, with 8 of 137 (5.8%) milk samples testing positive. Following administration of SICCT, a significant increase in the prevalence of ELISA positives was recorded on both test matrices (serum \( P < 0.001 \); milk \( P < 0.001 \)). The highest recorded prevalence of positive results for both serum and milk samples was 39% (Figure 6.1). No statistically significant difference (\( P = 0.668 \)) was recorded in the prevalence of serum positive results, pre- and 72 hours post-SICCT. Similarly no statistically significant difference (\( P = 0.197 \)) was recorded in S/P ratios of serum ELISA results pre- and 72 hours post-SICCT. Both Box plots and GEE analysis highlight an increase in both
serum and milk S/P% readings subsequent to the 72 hour sampling (Figures 6.2 and 6.3; Table 6.2 and 6.3, respectively).

Statistically significant differences between pre- and post- SICCT milk ELISAs were recorded until 43 days post-administration of PPD, examined as both a continuous and categorical variable (Table 6.2). The prevalence of ELISA serum positive samples was not statistically different from pre-SICCT levels by day 58, while serum ELISA S/P ratios remained significantly elevated for 71 days post-SICCT (Table 6.3). It should be noted that a significant elevation in S/P ratios post-SICCT was again noted in November (trial day 143) for both milk and serum samples (Table 5.2, Table 5.3). No significant second level interactions were identified between independent variables.

Spearman correlation analysis of serum and milk samples generated pre SICCT values of $r_s 0.73$. Post SICCT values ranged from $r_s 0.55$ to $0.79$ with the highest levels recorded at post SICCT test 1 ($r_s 0.77$) and post SICCT test 6 ($r_s 0.79$).
Table 6.2. Multivariable GEE analysis of milk ELISA as a continuous (S/P % ELISA readings) and categorical (milk ELISA MAP positive/negative) dependent variable and independent variables

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Continuous variable (S/P % ELISA readings)</th>
<th>Categorical variable (Milk ELISA MAP positive/negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>P Value significant: 95% C.I.</td>
</tr>
<tr>
<td>Milk ELISA</td>
<td></td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Independent variable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time point</td>
<td></td>
<td></td>
</tr>
<tr>
<td>June 20 vs. May$^a$</td>
<td>17.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>July 11 vs. May</td>
<td>5.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>July 24 vs. May</td>
<td>0.29</td>
<td>0.850</td>
</tr>
<tr>
<td>August 8 vs. May</td>
<td>0.94</td>
<td>0.537</td>
</tr>
<tr>
<td>August 21 vs. May</td>
<td>0.42</td>
<td>0.784</td>
</tr>
<tr>
<td>Oct vs. May</td>
<td>1.51</td>
<td>0.322</td>
</tr>
<tr>
<td>Nov vs. May</td>
<td>5.65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^b$ vs. 2</td>
<td>-11.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2 vs. 3</td>
<td>11.2</td>
<td>0.001</td>
</tr>
<tr>
<td>2 vs. 4</td>
<td>8.3</td>
<td>0.002</td>
</tr>
</tbody>
</table>

$^a$May is the ELISA sample taken pre SICCT.$^b$Parity 1: 1st lactation. No significant interactions identified with other independent variables. C.I., confidence interval. Coefficient, difference across the sample population. Statistically significant P values highlighted in bold.
Table 6.3. Multivariable GEE analysis of serum ELISA as a continuous (S/P % ELISA readings) and categorical (serum ELISA MAP positive/negative) variables.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Coefficient</th>
<th>P value significant:</th>
<th>95% C.I.</th>
<th>Odds ratio</th>
<th>P value</th>
<th>95% C.I.</th>
<th>Model (P value &lt;0.001)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ELISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time point</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>June 14 vs. May</td>
<td>4.4</td>
<td>0.197</td>
<td>-2.3, 11.0</td>
<td>1.1</td>
<td>0.668</td>
<td>0.6, 2.1</td>
<td></td>
</tr>
<tr>
<td>June 25 vs. May</td>
<td>33.8</td>
<td>&lt;0.001</td>
<td>272, 40.5</td>
<td>10.7</td>
<td>&lt;0.001</td>
<td>6.1, 18.8</td>
<td></td>
</tr>
<tr>
<td>July 11 vs. May</td>
<td>37.9</td>
<td>&lt;0.001</td>
<td>313, 44.6</td>
<td>6.4</td>
<td>&lt;0.001</td>
<td>3.7, 11.1</td>
<td></td>
</tr>
<tr>
<td>July 24 vs. May</td>
<td>17.0</td>
<td>&lt;0.001</td>
<td>10.3, 23.7</td>
<td>2.3</td>
<td>0.004</td>
<td>1.3, 3.9</td>
<td></td>
</tr>
<tr>
<td>August 8 vs. May</td>
<td>8.7</td>
<td>0.010</td>
<td>2.1, 15.4</td>
<td>1.3</td>
<td>0.392</td>
<td>0.7, 2.3</td>
<td></td>
</tr>
<tr>
<td>August 21 vs. May</td>
<td>1.8</td>
<td></td>
<td>-4.9, 8.4</td>
<td>1.1</td>
<td>0.659</td>
<td>0.5, 1.8</td>
<td></td>
</tr>
<tr>
<td>September 5 vs. May</td>
<td>4.0</td>
<td>0.241</td>
<td>-2.7, 10.6</td>
<td>1.0</td>
<td>0.998</td>
<td>0.5, 1.8</td>
<td></td>
</tr>
<tr>
<td>October 1 vs. May</td>
<td>6.0</td>
<td>0.080</td>
<td>-0.7, 12.6</td>
<td>0.9</td>
<td>0.641</td>
<td>0.5, 1.6</td>
<td></td>
</tr>
<tr>
<td>November 1 vs. May</td>
<td>11.1</td>
<td>0.001</td>
<td>4.5, 17.7</td>
<td>1.3</td>
<td>0.392</td>
<td>0.7, 2.3</td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 vs. 2</td>
<td>-29.4</td>
<td>0.006</td>
<td>-50.4, -8.4</td>
<td>0.4</td>
<td>0.053</td>
<td>0.2, 1.0</td>
<td></td>
</tr>
<tr>
<td>3 vs. 2</td>
<td>-27.6</td>
<td>0.015</td>
<td>-50.0, -5.3</td>
<td>0.3</td>
<td>0.018</td>
<td>0.1, 0.8</td>
<td></td>
</tr>
<tr>
<td>4 vs. 2</td>
<td>-10.0</td>
<td>0.296</td>
<td>-28.8, 8.8</td>
<td>0.5</td>
<td>0.047</td>
<td>0.2, 1.0</td>
<td></td>
</tr>
<tr>
<td>Calving Date</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>February vs. January</td>
<td>-4.0</td>
<td>0.677</td>
<td>-22.9, 14.9</td>
<td>0.3</td>
<td>0.003</td>
<td>0.1, 0.6</td>
<td></td>
</tr>
<tr>
<td>March vs. January</td>
<td>-24.0</td>
<td>0.066</td>
<td>-49.7, 1.6</td>
<td>0.2</td>
<td>0.002</td>
<td>0.1, 0.5</td>
<td></td>
</tr>
<tr>
<td>April vs. January</td>
<td>-15.9</td>
<td>0.304</td>
<td>-46.3, 14.5</td>
<td>0.4</td>
<td>0.145</td>
<td>0.1, 1.4</td>
<td></td>
</tr>
<tr>
<td>February vs. March</td>
<td>21.7</td>
<td>0.036</td>
<td>1.4, 41.9</td>
<td>1.7</td>
<td>0.241</td>
<td>0.7, 4.4</td>
<td></td>
</tr>
</tbody>
</table>

May is the ELISA sample taken pre SICCT test.
Parity 1 – 1st lactation. No significant interactions identified between independent variables. C.I., confidence interval. Coefficient, difference across the sample population. Statistically significant P values highlighted in bold.
Figure 6.1. Percentage (%) of the herd testing positive on Johne's disease ELISAs (milk and serum) at different trial days, both pre and post the administration of the TB test. An increased number of positives are identified post TB test administration.
**Figure 6.2.** Box plot identifying differences in serum ELISA S/P ratios at different sampling points, both pre and post the administration of the TB test.

**Figure 6.3.** Box plot identifying differences in milk ELISA S/P ratios at different sampling points, both pre and post the administration of the TB test. To improve visualization of interquartile ranges, only S/P values <150 shown.
6.4.3 Faecal Samples

Weekly faecal culture of consistently ELISA positive cows yielded negative results. A total of ten animals yielded PCR positive results, two of which recorded positive results at each sampling time point. All PCR positive cows had recorded ELISA positive results prior to the SICCT. Veterinary examination did not yield any clinical signs of JD in these animals.

6.5 Discussion

The Irish cattle population is subjected to a comprehensive compulsory bTB eradication programme, involving administration of the SICCT on at least an annual basis (Good et al., 2007). The purpose of the current study was to investigate the impact of SICCT (i.e. administration of bPPD and aPPD) on both the within-herd prevalence of positive cows and ELISA S/P% ratios in an Irish dairy herd. The results of the current study can provide useful guidance to farmers and veterinarians on the optimum period to conduct MAP ELISA testing in regions engaging in comprehensive testing for bTB using SICCT.

Mycobacterial PPD provides antigenic stimulation to identify an animal whose immune system has been sensitised by infection with *M. bovis* or by exposure to cross-reacting antigens (de la Rua-Domenech et al., 2006). Intradermal administration of PPD elicits a delayed-type hypersensitivity response in sensitised individuals, a response mediated by T-cells. Two international studies, one conducted in Brazil (Varges et al., 2009), and the second in the UK (May et al.), have previously shown that tests for bTB involving intradermal administration of PPD, interfere with MAP ELISA diagnostics. Whether the increased number of ELISA positives identified post administration of PPD is due to increased Se or decreased Sp is unknown. Varges et al. (2009), suggests this PPD-induced immune response results in the production of cross-reacting antibodies detectable by MAP ELISA and leads to a reduction in MAP ELISA test specificity (Varges et al., 2009). If however the PPD induced T cell response assisted in activating B cells with subsequent antibody production, the post PPD period may reflect enhanced ELISA Se. The nature of
antibody/antigen binding post-SICCT requires thorough investigation as if results from this study were proved to measure an indication of increased sensitivity to MAP infection it may be advisable to perform MAP diagnostics under the period of the bTB test influence, rather than avoiding it as is currently recommended.

**Figure 6.4.** Variation in period of influence of SICCT in present study compared to Varges et al. 2009 is shown.

V1–V3; Approximate S/P results of positive cows identified using “in-house” ELISA by Varges et al. 2009. Current; mean ELISA S/P results from entire herd in the present study. Insert; a schematic of primary and secondary/memory immune response [adapted from Tizard 2013].

ELISA interference reported by Varges et al. (2009) occurred between 30 and 90 days post-PPD administration. Contrasting to the present study which documents the rising ELISA S/P of the herd (box plots), only the ELISA S/P of the individuals that recorded MAP positive results are included by Varges et al. Using a commercially available MAP ELISA (Pourquier), that study recorded
5/63 animals as MAP ELISA positive post PPD administration. Similarly the current study also recorded a significant increase in the S/P ratio and prevalence of ELISA positive animals post PPD administration. The increase and subsequent return to pre-SICCT prevalence however, occurred approximately 2 weeks earlier than the period of interference outlined by Varges et al. (2009). A number of reasons may account for this difference in findings across both studies. Contrasting to Varges et al. (2009) the current study herd had a history of recording serum MAP ELISA positive prior to PPD administration. It is possible, therefore, that cows tested in the current study had been pre-sensitised to MAP or additional mycobacterial-related antigens, therefore the more rapid immune response would be expected, indicative of a secondary humoral memory response (Nielsen et al., 2002a). The longer duration taken to record an IgG response and the lower percentage of positive cows (7.93%) identified post-PPD administration by Varges et al. (2009) may be indicative of a slower primary immune response (Figure 6.4).

Varges et al. (2009) examined both the single intradermal and comparative bTB test during their experiment, the routine screening test conducted in Brazil however for bTB is the caudal fold test, involving bPPD only. It is possible therefore, that the cattle examined by Varges et al. (2009) have never been sensitised to Mycobacterium avium including a lack of exposure to aPPD for bTB testing. It should be noted that administration of aPPD alone however did not result in an increase in ELISA positives (Varges et al., 2009). This may suggest that bPPD may be responsible for interference in both studies. Different ELISA kits were used by each study for MAP antibody detection. Different tests use different antigens, however superiority of immunogenicity and cross reactivity has not been evaluated. Nielsen and Toft (2008) suggest that cross reactivity may be specific to certain geographical areas. This may explain differences between both study results. Further research should investigate if different test antigens show comparable results following stimulation with PPD. The inclusion of a greater number, and diversity, of animals managed under similar conditions would also strengthen findings.

In Ireland, herds restricted due to a positive bTB diagnosis (Directive 64/432/EEC), undergo two repeat tests at a 60-day interval. For herds operating
under these restrictions, the results of the current study highlight that milk samples may be a more suitable test matrix than serum ELISA to avoid test interference. The shorter interval for milk to return to pre SICCT levels may reflect the difference in IgG sub-classes (IgG1 subclasses predominate in bovine milk, but only constitutes approximately 50% of serum IgG) between serum and milk with a lower response by milk IgG populations against MAP (Lombard et al., 2006). Although May et al., recorded significantly higher milk ELISA readings 4.5 weeks post-PPD administration, only a single time point post-SICCT was examined. The period of bTB interference recorded by the current study and May et al. do overlap, however the limited sampling and statistical analysis outlined by May et al., present difficulties in allowing direct comparison of both datasets. The fact that a number of regions in the UK administer the SICCT on one occasion every four years (DEFRA, 2012) a much longer testing interval than experienced by Irish herds, also present difficulties in this regard. The potential for a cumulative effect of PPD administration from multiple bTB tests over a number of years, therefore, requires thorough investigation to fully characterise the impact of SICCT on MAP ELISA testing.

Ireland reports a relatively low prevalence of JD ELISA positives (Good et al., 2009, Nielsen and Toft, 2009) and clinical cases (Barrett et al., 2006) in comparison to other countries. Given that environmental conditions in Ireland are conducive to the growth of mycobacteria (Cooney et al., 1997), and that Irish farmers engage in high risk management practices with regard to spread of JD e.g. widespread pooling of colostrum (Cashman et al., 2008)( Kennedy et al, 2014), a higher prevalence of clinical cases might be expected. Additionally, an Irish study (Hoogendam et al., 2009) recorded no significant effect of MAP sero-status on milk production parameters contrasting with international studies (Ott et al., 1999, Hasonova and Pavlik, 2006). It is possible that the high levels of environmental mycobacteria in Ireland (Cooney et al., 1997) yield cross-protection against MAP, and/ or may also be responsible for the suggested memory response detected by MAP ELISA in the current study. It has previously been reported that exposure to environmental mycobacteria may give a low level of protection against *M. tuberculosis* (Orme and Collins, 1984, Orme et al., 1986). Hope et al. (2005) reported low level protection against *M. bovis* following exposure to *M. avium*, and that pre-exposure to *M. avium* results
in an imprinting of memory against avian antigens onto T-lymphocytes. Combining an amnestic response to environmental mycobacterial infection with continuous boosting of T cell responses, as part of the cell mediated delayed type hypersensitivity response induced post PPD administration, may contribute to disease control at the animal level. Based on the increase in antibody titres in MAP ELISA recorded post-PPD administration, it is our hypothesis that repeated annual administration of aPPD and bPPD may induce a vaccine-like effect lessening the clinical manifestations of MAP infection. Caution should be exerted as this hypothesis is not based on experimental infection with corresponding reduction in clinical JD signs. As however control of MAP infections is associated with a T cell response (Stabel, 2000), this hypothesis requires in depth investigation as to whether the increase in antibody levels post-PPD administration equates to an increased T-cell response that would prove effective against MAP.

An advantage of the current study was the use of a compact spring-calving herd. This ensures that all cows examined were at a similar stage of lactation and physiological status. In agreement with a previous study (Lombard et al., 2006), cows in late lactation were more likely to yield a MAP ELISA positive result using milk samples, due to a lessening of the dilution effect of milk yield on antibody levels (Nielsen and Toft, 2012). Interestingly, an increase in the prevalence of serum ELISA positives was also recorded in late lactation in agreement with a Danish study (Nielsen et al., 2002a). The increase in prevalence of serum ELISA positives in the current study correspond with housing which may increase the likelihood of exposure to mycobacterial antigens by increasing the potential for faecal contact. Nielsen et al. (2002a), showed parity 2 and greater to be more likely to test ELISA positive relative to parity 1 cows which is also highlighted in the current study. Parity 3 and 4 animals, however, were in general less likely to test positive than parity 2. The majority of Irish farmers target compact calving seasons (ICBF, 2014) and strict culling practices are often in place (Maher et al., 2008). These culling practices may lead to less ELISA positive animals remaining in the system post second lactation. Results from this study indicate that age of animal at sampling and
timing of JD ELISA tests relative to stage of lactation and time of bTB testing, are important considerations when interpreting ELISA results.

6.6 Conclusions

Administration of PPD as part of the bTB test corresponds to an increased prevalence of ELISA positives for JD. Diagnostic sampling for JD utilising milk ELISA should be avoided in the 43 day period following the bTB test, with serum ELISA sampling not recommended for an additional 28 days. Future work will involve investigation as to whether the increase in antibody levels post-PPD administration equates to an increased T-cell response that would prove effective in lessening the clinical manifestations of MAP infection.
Chapter 7: Investigations and implications of associations between mycobacterial purified protein derivative hypersensitivity and MAP-antibody ELISA in Irish dairy cows.

DOI: http://dx.doi.org/10.1016/j.rvsc.2017.01.018
7.1 Abstract

Intradermal testing, involving administration of purified protein derivative (PPD), to elicit a delayed hypersensitivity (DTH) response, is used as a diagnostic tool for bovine tuberculosis (bTB) and to aid in the identification of exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne’s disease (JD). Further research is required to increase the diagnostic value of skin testing for MAP. The aim of this study was to investigate if animals showing DTH reactions to PPD had an associated increase in MAP ELISA response, thereby identifying potential cases of sub-clinical JD.

A 139-cow dairy herd was recruited to the study. During the mandatory annual bTB test, skin thickness measurements (mm) were recorded at the site of avian and bovine PPD administration. Cows were categorised based on recording no DTH, DTH at both PPD administration sites and DTH at one PPD site only.

Blood samples were collected pre and post bTB testing, and ELISA tested. Generalised estimating equations were performed to identify associations between DTH responses and MAP ELISA results. Significant associations were identified between PPD DTH responses and MAP ELISA readings. Animals with DTH at both avian and bovine PPD sites were most likely to test ELISA positive in the post-PPD period relative to other categories.

Further research is required to identify whether skin thickness increases post-PPD and associated increase in ELISA response, identifies animals previously exposed to MAP, or if results are due to cross reactivity.
Intradermal testing, involving administration of purified protein derivative (PPD), aids diagnosis of bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (de la Rua-Domenech et al., 2006). In countries where there is a possibility of co-infection with environmental mycobacteria, the single intradermal cervical comparative skin test (SICCT), involving administration of both avian PPD (PPDa) and bovine PPD (PPDb) at two separate injection sites, is used to improve test specificity (de la Rua-Domenech et al., 2006). The test elicits a delayed type hypersensitivity (DTH) response in previously exposed animals. Antigens are transported to regional lymph nodes where memory T-cells are activated. These cells mediate an immune response, visible as an increase in skin thickness at PPD injection sites (de la Rua-Domenech et al., 2006). Interpretation of SICCT is based on comparative skin thickness measurements 72 hours post-PPD administration. An animal is classified a reactor if PPDb skin thickness measurements are 4mm or greater than PPDa measurements (de la Rua-Domenech et al., 2006).

Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), progresses slowly and is notoriously difficult to diagnose (Olsen et al., 2002). Based on the same test principle as SICCT for bTB, intradermal testing has also been used to detect previous exposure to MAP and identify potentially sub-clinical JD cases (Manning and Collins, 2001, Gilardoni et al., 2012). It is suggested that animals showing skin thickness increases at both inoculation sites, but a larger increase at the avian injection site are potentially sub-clinically infected with MAP (Gilardoni et al., 2012). Similarly, Manning and Collins (2001) suggest further monitoring of animals showing strong responses to PPDa with regard to MAP infection.

The World Organisation for Animal Health (OIE) has suggested the use of intradermal testing in certain situations to enhance MAP control programmes but has also stated that further research is required to increase the diagnostic value of skin testing (OIE, 2016). The aim of this study, therefore, was to investigate if animals showing DTH reactions to either PPDa or PPDb had an
associated increase in MAP ELISA response, thereby identifying potential cases of sub-clinical JD in Irish dairy cows.

7.3 Materials and methods

7.3.1 Study Herd

A 139-cow dairy herd was recruited to the study in 2014. This herd had tested bTB negative for 3 years. A clinical case of JD, confirmed by pathological examination, was recorded in May 2013. In August 2015, during routine herd screening, five cows tested ELISA positive. None showed clinical signs of JD. Three were MAP faecal culture positive; two were confirmed by pathological examination.

7.3.2 Comparative Skin Test

During the 2014 statutory SICCT, PPDa and PPDb skin thickness measurements (mm) were recorded for each cow (continuous variable for statistical analysis). Cows were also assigned to DTH categories (categorical variable for statistical analysis) based on the 72-hour post-PPD assessment. Cows were categorised based on showing no DTH response, DTH at both PPD sites and DTH at one PPD site only (≥1mm skin thickness increase was classified as a response).

7.3.3 Blood Sampling

Blood samples were collected immediately prior to administration of PPD. Samples were subsequently collected on days 10, 16, 42, 57, and 91 post-SICCT. Serum samples were tested using the ID Screen Paratuberculosis Indirect Test (ID Vet, Montpellier, France). Results were reported as sample to positive ratio (S/P ratio) (continuous variable). MAP-antibody status (positive vs. negative) was assigned according to kit manufacturer interpretation i.e. S/P ≥ 70 = Positive (categorical variable).
7.3.4 Statistical Analysis

A generalized estimating equation (GEE) was performed to identify associations between DTH response (millimetres) and MAP ELISA S/P ratio. A second GEE was conducted to identify associations between categorical DTH responses and MAP positive / negative status. Sampling time point, breed and parity were included as independent variables, with interactions examined. Variables were considered significant at the 5% level. A Gaussian distribution and an identity link function were used for continuous variable analysis. For categorical variable analysis, a binomial distribution and a logit link function were used. An autoregressive correlation was applied to both analyses to account for the expected correlation between consecutive measurements.

7.3.5 Faecal Sampling 2016

In June 2016, the entire herd (139 cows) was faecal sampled and PCR tested. The 2016 herd included 70 cows that had partaken in the initial 2014 study. PCR testing was conducted by a commercially accredited laboratory (Enfer Laboratories, Ireland). Samples were pooled in batches of five. All positive pools (three i.e. 15 cows) were subsequently tested individually. One month later repeat PCR testing was performed on any cow that had formed part of a positive pool or that had previously tested MAP ELISA positive (not under bTB test influenced period). Faecal culture was performed on animals with an ELISA positive or PCR positive result. This was conducted by the Central Veterinary Research Laboratory (Department of Agriculture Food and the Marine). Univariate analysis was performed to identify associations between recording a PCR positive result in 2016 and a post- SICCT ELISA positive result or DTH response in 2014.

7.4 Results

No bTB positive reactors were identified. All cows were MAP ELISA negative pre-PPD administration. On day 10 post-PPD, 30% (n=42) of the herd tested ELISA positive. A lower proportion of the herd recorded seropositive results
thereafter (27%, 12%, 3%, and 0% MAP ELISA positive at day 16, 42, 57, and 91, respectively).

Fifty cows recorded no DTH response. Thirty five cows recorded DTH responses at both PPDa and PPDb administration sites. The remainder of cows recorded DTH at the PPDa site only. The maximum increase in skin thickness at PPDa site was 17 mm. This cow recorded an increase of 3mm at the PPDb site. She remained ELISA negative throughout the duration of the study. The maximum increase in skin thickness at the PPDb site was 13 mm with this cow recording an increase of 15mm at the PPDa site. In the post-CST period this cow tested ELISA positive until day 91. She was culled for non-JD related reasons prior to herd PCR sampling. Of the three 2015 faecal culture positive cows, one showed DTH at the PPDa site only, the others recorded no DTH. None tested ELISA positive in the period post- SICCT.

Significant associations between DTH measurements and MAP S/P ratios are shown in Table 7.1. On day 10, a 1mm skin thickness increase at the PPDb administration site was associated with a MAP ELISA increase of 7.6 S/P ($p<0.001$). Similarly, at the PPDa administration site a 1mm skin thickness increase was associated with a MAP ELISA increase of 4.9 S/P ($p<0.001$). Analysis of categorical variables showed that animals reacting at both PPDa and PPDb administration sites were 3.9 times (odds ratio) more likely to test MAP ELISA positive than animals with no DTH response ($p=0.004$). Animals responding at the PPDa site only were not statistically more likely to test MAP ELISA positive than animals displaying no DTH ($p=0.391$). Box plots displaying S/P ratios of each DTH category are outlined in Figure 7.1. Thirty-nine cows who showed no DTH response, although remaining ELISA negative (S/P <70) throughout the study, recorded increased S/P ratios until day 42 post- SICCT. ($p=0.21$).

In 2016, of the remaining 70 cows from the initial study; three recorded a PCR positive result, of which one had tested ELISA positive in the 2014 post- SICCT period. Two had shown DTH at both PPD administration sites. All were culture negative. No significant association was identified between testing PCR positive in 2016 and DTH / ELISA results in 2014.
Table 7.1: Results from GEE analysis showing associations between DTH response (mm), at each PPD administration site, and MAP ELISA S/P ratio post-SICCT.

Only statistically significant results are shown (P <0.05).

<table>
<thead>
<tr>
<th>Time</th>
<th>Site of inoculation</th>
<th>PPD Coefficient</th>
<th>P Value</th>
<th>95% Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian</td>
<td>4.9</td>
<td>&lt;0.001</td>
<td>3.0, 6.7</td>
<td></td>
</tr>
<tr>
<td>Day 10 vs. Pre-SICCT</td>
<td>Bovine</td>
<td>7.6</td>
<td>&lt;0.001</td>
<td>3.9, 11.3</td>
</tr>
<tr>
<td>Avian</td>
<td>5.3</td>
<td>&lt;0.001</td>
<td>3.0, 7.8</td>
<td></td>
</tr>
<tr>
<td>Day 16 vs. Pre-SICCT</td>
<td>Bovine</td>
<td>5.2</td>
<td>&lt;0.001</td>
<td>0.3, 10.1</td>
</tr>
</tbody>
</table>

Cl: Confidence interval

*aCoefficient: represents the increase in MAP ELISA S/P ratio for every 1mm increase in DTH skin thickness response*
Figure 7.1: Box plots showing the differences in MAP S/P ratios between DTH categories at each sample date.
7.5 Discussion

SICCT has been used to aid identification of exposure to MAP, with studies suggesting that MAP positive animals will react at both PPDa and PPDb inoculation sites (Gilardoni et al., 2012). Furthermore as an anamnestic rise in humoral immune response post-CST has been used to increase bTB ELISA test sensitivity (Harboe et al., 1990, Casal et al., 2014). It was our aim to investigate if animals showing SICCT DTH reactions had an associated increase in MAP ELISA response, thereby identifying potential cases of sub-clinical JD. In agreement with studies showing improved bTB ELISA sensitivity post PPD administration (Harboe et al., 1990, Casal et al., 2014), results from this study showed increased identification of MAP ELISA positive animal’s post-SICCT. Further to this, cows showing DTH at both PPD administration sites recorded the highest odds ratio of testing MAP ELISA positive in the post-SICCT period. As a cow known to be shedding MAP was removed from this herd prior to study initiation, there is the possibility many of the herd were previously exposed to MAP and were potentially sub-clinically infected. Results from this study therefore indicate that combined use of DTH responses and anamnestic rise in ELISA response may improve identification of sub-clinical MAP infection.

Alternatively, however Varges et al. (2009) has suggested that increased MAP antibody titers post-PPD administration are due to cross reactivity from mycobacterial antigens i.e. a reduction in ELISA specificity. Possible evidence of decreased specificity in the current study includes cows that remained ELISA negative for the duration of the study and that showed no DTH response post-SICCT, recorded increased MAP ELISA S/P ratios. Additionally of the three cows with MAP faecal culture positive results in 2015 (and presumably sub-clinically infected at the time of CST in 2014), none reacted at both PPDa and PPDb administration sites or tested MAP ELISA positive post PPD administration, potentially indicating that combined use of DTH and anamnestic effect may not be an efficient method of identifying MAP infected animals.
Two years, a time period potentially allowing JD progression, lapsed between the initial study and PCR sampling. If initial study results were taken as an indicator of improved sensitivity the lack of significant association identified between PCR positive results and post-SICCT MAP ELISA or DTH responses was surprising. It should be noted however, given the limitations of JD diagnostics (Olsen et al., 2002), a negative PCR result does not preclude the possibility of sub-clinical MAP infection. Indeed a possible study limitation is that the herd had an annual cull rate of approx. 25% (in line with national data) and JD positive cows may have been culled prior to PCR testing. Prior to their culling however, none tested ELISA positive outside the period of bTB test influence and none showed clinical signs of JD, possibly again indicating combining both test methods may not be an efficient method of identifying MAP infected animals.

Chiodini et al. (1984) have suggested that some MAP infections are successfully eliminated by the immune system and it is known a strong cell mediated immune (CMI) response is required to control MAP infections (Stabel, 2006). This possibly indicates that in the current study, the combined test methods may truly have identified animals that were exposed to MAP, but if the animals showing strong DTH reactions, mounted a successful CMI response they may not necessarily progress to show signs of JD. Based on this it may prove more beneficial to use combined testing regimes to aid herd management in terms of colostrum and calving area management rather than as a tool to select animals for culling. As serology is the mainstay for many JD control programmes, until further research is conducted, it is advisable to avoid sampling for serology based MAP diagnostics for 71 days post-PPD administration (Kennedy et al., 2014a).

7.5 Conclusions

An association between PPDa & PPDb DTH and MAP ELISA results has been highlighted, with a greater response noted to PPDb. Further research is
required as results from this study cannot definitely state whether use of the combined methods results in improved test sensitivity or decreased specificity for identifying MAP infection. It is therefore advisable to adhere to the current recommendation (Kennedy et al., 2014a) to avoid sampling for serology based MAP diagnostics for 71 days post-PPD administration.
Chapter 8: Is TB testing lowering the risk of clinical Johne's disease in the Republic of Ireland?
8.1 Abstract

The Republic of Ireland reports a relatively low prevalence of Johne's disease (JD) compared to international counterparts. Postulated reasons for this include a lower average herd size and a grass-based production system. Ireland also engages in high levels of bovine tuberculosis (bTB) testing, however. As interferon-gamma (IFN-γ) is believed to play a key role in protecting against JD, it is our hypothesis that administration of purified protein derivative (PPD) is associated with a systemic increase in IFN-γ production which may potentially limit clinical progression of the disease.

In all, 264 cows were included in the study (201 Friesian and 63 'Non-Friesian'). As part of the compulsory annual bTB test, avian and bovine PPD were administered at two separate cervical sites. Repeated comparative measurements and blood samples were taken before PPD administration and at an interval of 72 hours. Plasma samples were tested using the Bovigam IFN-γ kit. Samples were assayed in duplicate and mean optical density values used for data analysis. Wilcoxon rank sum test was used to highlight significant differences between pre- and post-bTB test IFN-γ measurements. To investigate the impact of additional independent variables, generalized estimating equations were employed to examine associations between IFN-γ production, sampling time point, and additional variables.

IFN-γ production significantly increased at the post- bTB time point ($p<0.001$) which may indicate a role for bTB testing in controlling JD. Non-Friesians were found to record lower increases in IFN-γ production than Friesian cows post-bTB test ($p=0.037$) which may explain the increased risk of JD associated with Jerseys.
8.2 Introduction

Interferon gamma (INF-γ) is an inflammatory cytokine that is critical to both innate and adaptive immune systems (Schoenborn and Wilson, 2007) across mammalian species. It is an important activator of macrophages following bacterial exposure (Zurbrick et al., 1988). INF-γ in more recent decades has been applied as a tool in the diagnosis of mycobacterial diseases such as bovine and human tuberculosis (bTB and hTB) (de la Rua-Domenech et al., 2006, Horvat, 2015), Johne’s disease (JD) (Gilardoni et al., 2012), and leprosy (Manandhar et al., 2000).

Mycobacteria are a leading cause of debilitating infections in domesticated animals and wildlife (Rastogi et al., 2001). Certain mycobacteria also have public health implications including bovine tuberculosis (bTB). A compulsory eradication scheme was initiated in the Republic of Ireland in the 1960’s to both protect human health and agricultural exports (Good, 2006). Although levels of TB in the human population in Ireland have dramatically reduced, bTB eradication remains necessary in order to comply with European trading conditions (Directive 64/432 EEC)(Good, 2006).

Bovine tuberculosis (bTB) is caused by Mycobacterium bovis (M. bovis). In the late 1800’s Robert Koch identified the tubercle bacillus and also developed tuberculin, a glycerol extract of pure culture of tubercle bacilli (Kaufmann and Schaible, 2005). The intradermal skin test which utilises tuberculin has been used diagnostically for detection of human and bovine TB for over 100 years. Tuberculin or purified protein derivative (PPD) is produced by growing a mycobacterial organism on liquid culture, subsequently heat treating, filtering, washing, and then re-dissolving it into a sterile preparation free from intact mycobacteria (Monaghan et al., 1994, de la Rua-Domenech et al., 2006). When injected intradermally, PPD provides an antigen source to identify animals whose immune system has been sensitised by previous exposure to the
mycobacterium. Sensitisation in an animal is identified by development of an oedematous lesion at the site of injection (Figure 8.1).

Figure 8.1: Measurement of oedematous lesion at the site of PPD administration

Ante-mortem screening for bTB in Ireland is conducted using the single intradermal cervical comparative test (SICCT). All bovines over six weeks of age are tested a minimum of once per year and depending on results may undergo repeat testing up to five times in a single year (Good et al., 2007). While many countries use the single intradermal test, where *M. bovis* PPD is administered in isolation, the SICCT utilises intradermal introduction of both *M. bovis* (bPPD) and *M. avium* (aPPD) PPD at two different sites on the neck (de la Rua-Domenech et al., 2006). A relative difference in the size of the oedematous lesion of ≥4 mm at the bPPD injection site compared to aPPD site indicates a positive result (Monaghan et al., 1994).

The interferon-gamma (IFN-γ) assay is also approved by the EU for bTB testing (de la Rua-Domenech et al., 2006) and is used as an ancillary test in the Irish
bTB eradication scheme. The IFN-γ test involves incubation of heparinised blood samples in the presence of test antigens (avian and bovine PPD). Subsequently plasma supernatant from each blood aliquot is harvested. IFN-γ production is estimated using an enzyme immunoassay (EIA) (Rothel et al., 1990). IFN-γ testing can either be used to confirm bTB SICCT positive reactors or alongside intradermal tests to increase diagnostic sensitivity (Schiller et al., 2010a).

PPD administration has been shown to boost in-vitro IFN-γ production in the case of bTB (Whipple et al., 2001, Palmer et al., 2006). Ota et al. (2007), found skin testing alone significantly induced PPD-specific IFN-γ producing cells in humans. Furthermore a study by Thom et al. (2004) suggested that repeat skin tests for bTB in calves subtly interfered either positively (induced a protective effect) or negatively (induced a suppressive effect) on the hosts immune system, highlighting important interactions between PPD administration and host immune responses.

An additional pathogenic and speculated zoonotic member of the Mycobacteriaceae is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne’s disease (JD) in cattle (Sweeney et al., 2012). Currently there is no effective treatment for JD, and control is based on breaking the cycle of transmission to susceptible animals by limiting contact with MAP infected faeces, colostrum and milk (Garcia and Shalloo, 2015). It is generally believed that early subclinical MAP infections result in a cell-mediated immune response involving delayed type hypersensitivity (DTH) with production of cytokines by T-lymphocytes, including IFN-γ (Stabel, 2000, Coussens, 2004). Indeed, IFN-γ has been established as an important cytokine in host defenses against JD (Coussens, 2004).

Ireland reports a relatively low prevalence of clinical MAP and MAP sero-positivity (Good et al., 2009). Additionally, Kennedy et al. (2016) have
hypothesised that Irish cows may be less susceptible than international counterparts to developing clinical signs of JD. Reasons suggested for this include low average herd size (75 cows), predominantly pasture based management systems, and the comprehensive bTB testing regime conducted in Ireland (Kennedy et al., 2014a, Kennedy et al., 2016). In terms of investigating this further, we sought to examine the impact of administration of avian and bovine PPD, as part of the required annual SICCT test, on in-vivo levels of serum IFN-γ. Our overall objective was to elucidate whether bTB testing in Ireland could be contributing to MAP control/suppression in Irish herds.

8.3 Materials and methods

8.3.1 Study Herd

A total of 265 cows were recruited to the study in April 2016, from a farm that had been depopulated in 1997 following confirmation of a case of BSE. The current herd, therefore, consisted of descendants of cows used to repopulate the farm (O Farrell et al., 2001). Since establishment of the new herd in 2000, cows from 47 different herds of origin had been purchased into the herd. Breed, age, parity, MAP ELISA status, and bTB status was available for each cow. Approximately 85% of the herd were spring-calving (i.e. calving between 1st Jan and 30th April). Herd SICCT history was examined in advance of recruitment of the herd to the study. The herd had been enrolled in the voluntary Animal Health Ireland (AHI) pilot JD control programme since 2014. All animals were examined for clinical signs of JD prior to study initiation.

8.3.2 Single intradermal cervical comparative test (SICCT)

The routine annual SICCT was administered by the farm's private veterinary practitioner in May 2016. It was conducted in line with standard Department of Agriculture guidelines (DAFM, 2017). Briefly, on the middle one third of the neck, two injection sites (dorsal and ventral), 12.5 cm apart, were clipped and skin-thickness measurements recorded with callipers. McLintock syringes
(Duggan Veterinary, Ireland) were used to administer 0.1 mL avian PPD dorsally and 0.1 mL of bovine PPD at the ventral site. PPD was supplied by DAFM in line with statutory requirements. Seventy two hours post-PPD administration skin thickness measurements at both injection sites were re-assessed to evaluate the presence or absence of a DTH response.

8.3.3 Blood samples

Blood samples were collected pre- and 72 hours post-PPD administration. Pre-SICCT samples were tested for both IFN-γ and MAP serological response. The 72-hours post samples were tested for IFN-γ levels only. Finally, a blood sample was collected ten days post-SICCT to examine the serological response on MAP ELISA. Blood samples were taken from the coccygeal vein using 20 gauge needles into evacuated lithium heparin blood sampling tubes. Samples were centrifuged for 15 minutes at 3000g and serum aspirated for same-day testing.

8.3.4 MAP ELISA

Serum MAP ELISA tests were conducted by a commercial ISO17025 accredited laboratory (Enfer Laboratories, Co. Kildare), designated by AHI for the Irish voluntary JD control programme. Samples were tested using the ID Screen Paratuberculosis Indirect Screening Test (ID Vet, Montpellier, France). Results were reported as sample to positive ratios (S/P ratio) calculated using the formula S/P ratio = \((\text{OD Sample} - \text{OD Negative control}) / (\text{OD Positive control} - \text{OD Negative control}) \times 100\). The test is an \textit{M. phlei} absorbed ELISA which detects anti-MAP IgG. For the purposes of reporting within-herd MAP prevalence, ELISA S/P ratio results were categorised according to manufacture instructions i.e. samples recording S/P ratios of ≥70 S/P categorised as seropositive.
8.3.5 IFN-γ sample preparation and testing

Samples for IFN-γ were tested using a modified version of Bovigam IFN-γ kit (Celtic Diagnostics Ltd, Dublin 22, Ireland). The first step in this test typically involves a blood culturing step, involving addition of a negative control antigen, avian PPD, and bovine PPD to three separate aliquots of whole blood and incubated overnight. The second stage comprises measuring the production of IFN-γ from the stimulated lymphocytes in separated plasma using a monoclonal antibody based sandwich enzyme immunoassay (EIA). For our purposes, we used the intradermal administration of PPD to study cows as the lymphocyte stimulation step, and following centrifugation, assayed plasma directly using the IFN-γ EIA. This was deemed a suitable methodology as our purpose was to compare in-vivo IFN-γ levels pre- and post- PPD administration as opposed to identifying bTB infected cattle. All plasma samples for IFN-γ were plated within six hours of blood collection from cows.

Samples were assayed in duplicate and results reported as optical density values at 450nm (OD_{450}). The conventional kit interpretation is calculated by comparing mean negative control antigen, avian PPD and bovine PPD OD_{450} values. In the current study, we simply compared pre- and post-SICCT plasma IFN-γ levels as all cows were required to be administered with both avian PPD and bovine PPD. It should be noted that two ELISA plates recorded negative controls outside manufacturer recommendations (Recommended Mean Negative Control <0.13. The two plates recorded negative controls of 0.15 and 0.186 respectively). As the kit was not being used as conventionally specified it was decided to perform analysis including and excluding results from both plates (n=264 vs n=179 cows). Analysis yielded broadly similar results, analysis relating to breed differences however changed to recording a tendency (p=0.088) rather than statistical significance (P = 0.04). Results reported are from n=264 cows.
8.3.6 Cow classification

For SICCT, all cows were classified based on relative differences in skin thickness measurements at avian and bovine PDD injection sites. An animal displaying a 4mm or greater skin thickness increase at bovine compared to avian injection sites was classified as a positive reactor, an increase of ≥3mm and <4mm as inconclusive, and animals with lower or no increases in skin thickness measurements classified as negatives. In terms of MAP ELISA, manufacturer positive cut-off values were applied to classify cows as seropositive or seronegative. As a negative control antigen was not used for IFN-γ analysis, we established a positive-cut off based on the following formula; IFN-γ cut-off = mean (pre-SICCT OD450) + 3 standard deviations (pre-SICCT OD450). Cows yielding OD450 values greater than or equal to this cut-off value were classified as IFN-γ positive. Finally, cows were classified on the basis of breed (Holstein-Friesian, non-Holstein-Friesian) and parity (1 to 8).

8.3.7 Data Analysis

Descriptive analysis and dataset construction were completed in Excel (MS Office 2010). Normality of continuous datasets was examined visually using ladders of power histograms. Statistical analyses were completed using Stata version 12 (StataCORP, USA). Wilcoxon rank sum tests were used to investigate differences between pre- and post-SICCT IFN-γ, OD450 results, pre- and post-SICCT MAP ELISA results, avian PPD site measurements and bovine PPD site measurements (continuous variables). Univariable linear regression was initially used to examine the association between IFN-γ production (dependent variable) and sampling time point (pre-SICCT vs. post-SICCT), breed (Friesian vs. non-Friesian), parity (1, 2, 3, 4, 5, 6, 7, 8), changes in skin thickness measurement due to DTH pre- and post-avian PPD administration (continuous variable in mm) and skin thickness measurement due to DTH pre- and post-bovine PPD administration (continuous variable in mm). Difference between pre and post-SICCT MAP ELISA S/P ratio was also examined as an independent variable. A multivariable generalised estimating equation (GEE)
was subsequently built using independent variables which yielded P values ≤ 0.10 (Table 8.1) in univariable models.

A second GEE analysis was conducted to investigate associations between MAP ELISA S/P ratio (dependent variable) and the independent variables described previously, namely, sampling time point, breed, parity, and skin thickness measurements at avian and bovine PPD injection sites. Difference between pre and post-SICCT IFN-γ production was also examined as an independent variable. Both GEE models were constructed by backwards elimination with a forward step. Second level interactions between independent variables were examined and included in both models at $P \leq 0.05$. A Gaussian distribution and an identity link function were used. An exchangeable correlation was applied to both analyses.
Table 8.1: Univariable analysis examining associations between variables. *P*-values in bold highlight variables subsequently included in logistic regression models.

<table>
<thead>
<tr>
<th></th>
<th>Breed</th>
<th>Parity</th>
<th>Pre SICCT IFN</th>
<th>Post SICCT IFN</th>
<th>Pre SICCT MAP</th>
<th>Post SICCT Map</th>
<th>Avian response</th>
<th>Bovine response</th>
<th>IFN difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parity</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-SICCT IFN</td>
<td>0.76</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-SICCT IFN</td>
<td><strong>0.041</strong></td>
<td>0.84</td>
<td>0.63</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-SICCT MAP</td>
<td>0.41</td>
<td><strong>0.02</strong></td>
<td>0.12</td>
<td>0.31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-SICCT Map</td>
<td>0.21</td>
<td>0.96</td>
<td><strong>0.05</strong></td>
<td>0.77</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avian response (mm)</td>
<td>0.81</td>
<td>0.69</td>
<td>0.31</td>
<td>0.8</td>
<td>0.04</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine response (mm)</td>
<td>0.58</td>
<td>0.39</td>
<td>0.28</td>
<td><strong>0.08</strong></td>
<td>0.04</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Difference IFN Pre/Post SICCT</td>
<td><strong>0.06</strong></td>
<td>0.64</td>
<td><strong>&lt;0.001</strong></td>
<td><strong>&lt;0.001</strong></td>
<td>0.53</td>
<td>0.88</td>
<td>0.63</td>
<td><strong>0.05</strong></td>
<td></td>
</tr>
<tr>
<td>Difference MAP response</td>
<td>Pre/Post SICCT</td>
<td>0.29</td>
<td>0.39</td>
<td><strong>0.06</strong></td>
<td>0.89</td>
<td>0.12</td>
<td><strong>&lt;0.001</strong></td>
<td><strong>&lt;0.001</strong></td>
<td><strong>&lt;0.001</strong></td>
</tr>
</tbody>
</table>
8.4 Results

8.4.1 Herd Information

The study herd consisted of 202 Friesians or Friesian crosses and the remainder were predominantly Jersey or Jersey crosses and a small number of Norwegian red (21). Herd SICCT history revealed minimal issues with bTB (no reactors in 5 years). No animal displayed clinical signs of JD.

8.4.2 SICCT

A total of two animals were classified as inconclusive on SICCT but none were classified as bTB positive. Both inconclusive animals were subjected to repeat SICCT at a 60 day interval and tested bTB negative. In all, 91 animals exhibited DTH to aPPD and the maximum increase in skin thickness recorded at the avian PPD site post-SICCT was 11mm. With regard to bovine PDD, 36 animals recorded DTH against it, 35 of whom had also recorded avian PPD DTH.

8.4.3 IFN-γ

A single cow recorded a pre-SICCT IFN-γ measurement of 0.7405 and was therefore removed from the analysis as an outlier. Using remaining pre-SICCT IFN-γ results, an IFN-γ positive cut off value of OD_{450} ≥ 0.083 was calculated. The median pre-SICCT IFN-γ OD_{450} was 0.02 (range 0.001 - 0.0815). Post-SICCT IFN-γ measurements ranged from 0.001 to 0.507 (Median: 0.05) (Figure 8.2). Seventy one animals were categorised as positive. Post-SICCT IFN-γ measurements were significantly higher than pre-SICCT OD_{450} values (P<0.001). Similarly, the multivariable GEE analysis highlighted a statistically significant increase in IFN-γ production post-SICCT (Table 8.2). A significantly lower increase in IFN-γ production post-SICCT was recorded in non-Holstein-Friesian compared to Holstein-Friesian cows (P = 0.037) (Table 8.2).
### Table 8.2: Significant associations between IFN-γ production and independent variables

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Coefficient</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
<th>Model (Model P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ Production</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-SICCT vs. Pre-SICCT</td>
<td>0.05</td>
<td>&lt;0.001</td>
<td>0.04, 0.06</td>
<td>Test Time point, Breed, (P: &lt;0.001)</td>
</tr>
<tr>
<td>Non- Friesian vs. Friesians</td>
<td>-0.01</td>
<td>0.037</td>
<td>-0.02, -0.001</td>
<td></td>
</tr>
</tbody>
</table>

### Table 8.3: Significant associations between MAP ELISA response and independent variables

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Coef</th>
<th>P Value</th>
<th>95% Conf. Interval</th>
<th>Model (Model P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP ELISA Response</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-SICCT vs. Pre-SICCT</td>
<td>33.9</td>
<td>&lt;0.001</td>
<td>28.5, 39.4</td>
<td>Test Time point, Avian PPD DTH, Bovine PPD DTH (P value: &lt;0.001)</td>
</tr>
<tr>
<td>Avian PPD DTH response (mm)</td>
<td>3.4</td>
<td>&lt;0.001</td>
<td>1.7, 5.0</td>
<td></td>
</tr>
<tr>
<td>Bovine PPD DTH response(mm)</td>
<td>4.4</td>
<td>0.013</td>
<td>0.1, 7.9</td>
<td></td>
</tr>
</tbody>
</table>
Figure 8.2: Box plots showing IFN-γ and MAP ELISA response both pre and post-SICCT.
8.4.4 MAP ELISA

Prior to the administration of avian and bovine PPD, six animals were classified as MAP ELISA positive with S/P ratios ranging from 72 to 189 (median = 97.1). Post-SICCT, 64 animals were classified as MAP ELISA positive, with a median S/P ratio of 112.6 (range 70 - 229).

A significant increase in MAP serological response was identified post-SICCT compared to pre-SICCT response (P<0.001) (Figure 8.2). A significant association was identified between MAP S/P ratio and both avian and bovine PPD DTH response. For every 1mm increase in bovine PPD DTH, an increase in MAP S/P ratio of 4.4 was identified (P = 0.013). Similarly for every 1mm increase in avian DTH an increase in MAP S/P ratio of 3.4 was recorded (P <0.001) (Table 8.3). No association was highlighted in this study between post-SICCT IFN-γ and antibody production (Figure 8.3).

![Figure 8.3](image)

**Figure 8.3:** No significant association was identified between post-SICCT IFN-γ production and post-SICCT antibody production. IFN-γ results*1000 to aid visualization.
8.5 Discussion

The primary aim of this study was to investigate if the administration of PPD during the routine annual SICCT for bTB was associated with an increase in circulating plasma IFN-γ. The significant association highlighted between PPD administration and an increase in systemic IFN-γ provides support for the theory that SICCT may have a potential immuno-protective influence in Irish cattle with regard to MAP infections and may contribute to the low levels of overt clinical signs of JD experienced on Irish farms (Good et al., 2009, Kennedy et al., 2014b).

IFN-γ, originally called macrophage activating factor (Schroder et al., 2004), orchestrates a diverse array of cellular activities, including up regulation of pathogen recognition, antigen processing, and antigen presentation. During infection, IFN-γ induced actions result in heightened immune system function and surveillance, with IFN-γ amplifying immune system response to pathogens (Schroder et al., 2004). IFN-γ has been established as an important cytokine in defenses against mycobacterial disease (Cooper et al., 1993, Flynn et al., 1993, Coussens, 2004) and appears important in limiting infection by MAP (Koets et al., 2015). In human medicine, IFN-γ has been used as a therapeutic adjuvant in wide range of diseases from atopic dermatitis (Hanifin et al., 1993) to ovarian cancer (Marth et al., 2006). Marciano et al. (2004), found that prophylactic use of IFN-γ was effective in patients with Chronic Granulomatous Disease. Given the role of IFN-γ in limiting progression of MAP infections (Koets et al., 2015) and its use as a therapeutic adjuvant and prophylactic agent, the increased level of IFN-γ recorded post-PPD administration in the current study may indicate TB-testing can contribute to MAP control in Irish dairy cows. As some animals in Ireland face up to five tests in one year (Good et al., 2007), the suggested protective effect of TB-testing may help limit progression of MAP infections.
Conflicting reports exist in the literature relating to PPD administration stimulating increased production of IFN-\(\gamma\) in vitro. Many studies report no significant effect observed after PPD administration (Doherty et al., 1995, Gormley et al., 2004a, Coad et al., 2010), while others (Whipple et al., 2001, Palmer et al., 2006) found increased IFN-\(\gamma\) responses. On reviewing relevant literature, Schiller et al. (2010b) found it could not be disregarded that skin testing induces an IFN response particularly in animals sensitised to environmental mycobacteria. It has been reported by Buddle et al. (2002), that many animals are naturally sensitised to environmental mycobacteria at a young age and develop an immunological response to such antigens by six weeks of age. Ireland is recognised as having an abundance of environmental mycobacteria (Cooney et al., 1997). As Irish livestock systems are largely grass-based with animals only housed for two to three months per year, potential exposure to such mycobacteria is highly probable. It is possible; therefore, that prior infection with environmental mycobacteria is contributing to the significant increase in IFN-\(\gamma\) response post-SICCT recorded in our study. Hope et al. (2005) also reported prior exposure to \textit{M. avium} induces low level protection to \textit{M. bovis} and may prime host immune responses. As to whether this could be extrapolated to environmental mycobacteria inducing low level protection against MAP in Irish cattle requires further investigation, but deserves serious consideration.

Vaccines have been available for JD since the early 20\textsuperscript{th} Century. The majority of vaccines are based on killed mycobacterium cells in an oil based adjuvant. Use is prohibited in a number of countries due to interference with TB diagnostics (Bastida and Juste, 2011). If results in the current study could be expanded upon and it was shown that PPD was exerting a vaccine like effect it may highlight a new control approach for areas not currently engaging in bTB testing programmes to a similar extent as Ireland. As clinical JD does occur in Ireland, PPD does not provide complete protection. Few commercial vaccines however, will report 100\% efficacy (Bastida and Juste, 2011). Research is required to see if a modified PPD preparation and administration protocol could
provide a comparable response to disease as provided by commercial vaccines.

An interesting finding from this study is that non-Holstein-Friesian breeds, consisting predominantly of Jersey and Jersey cross cows, produced significantly less IFN-γ in the post-PPD period than Holstein-Friesian and crosses. A number of studies have reported an increased likelihood of Channel Island breeds testing MAP positive (Withers, 1959, Çetinkaya et al., 1997, Sorge et al., 2011). Verschoor et al. (2010), reported differing expression of genes related to immune response and antigen processing in MAP infected Friesians versus Jerseys and Ballou (2012) demonstrated reduced immune responses to *E-coli* infection in Jersey calves compared to Holstein-Friesians. More specifically, of four MAP faecal culture positive animals detected in a single Irish mixed-breed herd over a five year period, all were of Jersey ancestry (Chapter 5). It is possible, therefore, that Channel Island cow breeds have a lesser ability to mount an immune response effective at clearing MAP infection. From a purely practical point of view, more stringent MAP-related management practices should be considered in herds containing Channel Island breeds to minimize JD transmission. Such an approach could also include a more severe interpretation of ELISA results during MAP herd surveillance. As the heritability of MAP susceptibility in Jersey cows has been reported at between 8–27% (Zare et al., 2014), an appropriate breeding programme could also greatly assist in improving MAP outcomes in Jersey cows.

In agreement with previous studies (Varges et al., 2009, Kennedy et al., 2014a), administration of PPD was associated with an increase in MAP antibody response. An association was likewise identified in this study between DTH skin reactions and MAP ELISA antibody response in agreement with a previous study (Kennedy et al., 2017). Interestingly Hostetter et al. (2005) reported that given the appropriate environment, opsonisation of MAP with specific antibodies can lead to an oxidative burst and reduced survival of MAP. Potentially this may
indicate that MAP antibodies produced post administration of SICCT (even as a result of cross reactivity), may contribute to the control of MAP. Further research would be required however as other researchers have reported the ineffectiveness of antibodies in controlling MAP.

The relatively low prevalence of MAP in Irish herds may be attributable to a number of factors. Previously suggested reasons including outdoor grazing systems and a lower average herd size compared to international counterparts (Kennedy et al., 2016). It has also previously been suggested that TB- testing may contribute to JD control, as MAP infected animals can show false positive reactions to SICCT, and be removed from the herd. This study is the first of our knowledge to report an alternative way bTB-testing may contribute to MAP control in Ireland and in this study we investigate if PPD can induce production of cytokines known to be immunologically important in the control of JD. Results are suggestive that bTB-testing may be contributing to JD control and requires comprehensive investigation. This current study however involves a single herd on one test date and more comprehensive studies are required to further investigate our hypothesis.

Although there was an increase in both post-SICCT IFN-γ and antibody production, no association was highlighted in this study between IFN-γ and antibody production. As Mikkelsen et al. (2009) reports that cell mediated responses can control or eradicate MAP, it may indicate that the animals with increased IFN-γ production in the current study are limiting the progression of the disease, and not producing antibodies. Surprisingly no association was identified between systemic IFN-γ production and DTH responses. Over 15 different cytokines however have been identified at the local site of tissue inflammation (Black, 1999). In humans it is known that TNF-alpha is more effective in inducing a local DTH response (Hernandez-Pando and Rook, 1994, Black, 1999). As a number of cytokines are involved in the DTH reaction not just IFN-γ it may explain the lack of association between IFN-γ production and DTH responses.
8.6 Conclusion

We can conclude from this study that administration of PPD as part of the bTB test is associated with an increase in MAP antibody response. IFN-γ production significantly increased at the post- bTB time point ($p<0.001$) which may indicate a role for TB testing in controlling JD.
Chapter 9: Summary, Conclusions and Implications
9.1 Thesis main findings

Control of JD at farm level is important, both in terms of animal health and welfare, and in certain cases, farm profitability (Garcia and Shalloo, 2015). Furthermore, confirmation of potential zoonotic links between MAP in milk causing Crohn's in humans could have significant implications for the global dairy industry (Groenendaal and Galligan, 2003), necessitating the implementation of control programmes. Rather than instigating a reactive approach, it is imperative Ireland adopts a proactive approach to initiating control programmes, in order to minimise MAP levels in our milk, especially given the high volume of Irish milk that is directed towards the baby formulae market (International Dairy Federation, 2013). Much scientific data has been gathered as part of this thesis and results will greatly contribute to our knowledge of the JD processes occurring in Ireland. Ultimately the knowledge resulting from this thesis will assist MAP diagnosis and control levels of JD in Ireland, thus improving animal welfare and contributing to the competitiveness of the Irish dairy industry.

Fortunately in Ireland we currently report a relatively low prevalence of MAP compared to international counterparts (Nielsen and Toft, 2009, McAloon et al., 2016a). Surprisingly given this low prevalence, thesis results showed that farmers are engaging in high risk management practices known to increase the risk of transmitting the disease. The results have highlighted that education is required among Irish farmers to promote the use of best practice management in order to limit JD transmission. AHI recently implemented a pilot JD control programme, which through the medium of VRAMPS allows participating farmers to highlight specific management changes that need to be implemented on their farms to minimise JD transmission. Results provided as part of this thesis provide baseline data of management practices occurring nationally and will allow benchmarking of the success of AHI's VRAMP programme. As management changes are commonly identified as the most critical element of JD control programmes (Groenendaal et al., 2002, McKenna et al., 2006), it will be essential that the practices identified in this study are remedied.
A previous study conducted in Ireland failed to identify a statistically significant association between ELISA positive status and production measures in cows sub-clinically infected with MAP (Hoogendam et al., 2009). Given the high level of JD associated management practices identified in study one, combined with the general view that JD has been increasing in Ireland in the past decade (Good et al., 2009, McAloon et al., 2016a) we expected to show an association between JD sero-prevalence and production parameters. Furthermore as large herd size is a known risk factor for testing JD positive (Barrett et al., 2011), we examined herds larger than the national average to maximise our chances of identifying such an association. Surprisingly, even though a greater number of ELISA positive cows were identified in the current study compared to Hoogendam et al., (2009) our results again indicated no significant association existed between MAP ELISA status and production parameters. A suggestion arising from this thesis is that in future studies, it may prove more beneficial to use faecal based diagnostic methods rather than ELISA when attempting to identify economic losses associated with JD in Irish herds. Additionally, as production losses are often used as a motivator to engage in control programmes (Bennett, 2003, Stott et al., 2005, Stott et al., 2012), the lack of association between MAP sero status and production parameters indicates that alternative motivation strategies will be required to stimulate interest in JD control. In this regard, potential areas of further research include examining the benefits to calf health or improved mastitis control, through the implementation of hygienic JD management practices. Maintaining motivation amongst farmers participating in JD programmes can be difficult (Ritter et al., 2017). Therefore identifying multiple benefits will potentially aid uptake and continued participation in JD control programmes.

An important component of this thesis was to implement an appropriate diagnostic and control programme for JD on an Irish dairy farm. Based on post mortem findings, we can conclude that unnecessary culling may result if animals are selected on the basis of ELISA testing alone. Combining ELISA results with faecal culture or PCR is advisable to enhance confidence that animals selected for culling are in an advanced stage of the disease. Overall results from this study imply that JD can be successfully controlled on Irish dairy
farms. As eradication was not achieved however, results highlight the long term commitment required when entering a JD control programme.

The diagnostic difficulties associated with JD are heightened in countries like Ireland that engage in high levels of bTB testing. Studies in this thesis have highlighted the complex nature of the relationship between MAP ELISA diagnostics and the bTB SICCT. Definitive guidelines have been provided indicating when it is appropriate to collect milk and serum samples for MAP ELISA in the post bTB SICCT period. These results will be invaluable to laboratories, veterinary clinicians and farmers. The increased ambiguity pertaining to MAP ELISA results post-SICCT can now be minimised through following time guidelines provided in this thesis and will increase confidence among vets and farmers that MAP ELISA results are not arising from SICCT interference.

Significant associations were identified between DTH reactions and post-SICCT ELISA response, it remains unclear, however, whether results are an indicator of improved test sensitivity or are a reflection of decreased specificity due to cross reacting antibodies. To determine if both tests can be used in combination to improve JD diagnosis will require further investigation. In the interim, it cannot be recommended to combine both test strategies to aid identification of subclinical JD infection.

A recurring question arising from the earlier studies in this thesis was: given Irish farmers engagement in high risk management practices why is the prevalence of JD in Ireland relatively low and why do we see relatively low numbers of clinical cases? A hypothesis that was formulated following our bTB SICCT and MAP ELISA investigations was that bTB testing was potentially contributing to MAP control in Irish dairy cows. Our results showed that following the administration of PPD as part of the SICCT there was an increase in the production of IFN-gamma. This cytokine is known to play an important part in the early control of JD. Our results provide an exciting topic of future research to identify if the bTB test is inducing a vaccine like effect limiting the advancement of clinical JD.
9.2. Conclusion

Generating scientific evidence to allow stakeholders in the Irish dairy industry to apply appropriate and effective disease control measures is essential. Given the variable progression of JD and the currently available diagnostics, JD diagnosis and control continues to pose a challenge. The results outlined in this thesis will however make an important contribution to Johne’s disease diagnosis and control in Ireland, and support AHI prioritised aims of minimising JD transmission in Irish cattle. Not only has this thesis highlighted the complex interactions between bTB and MAP diagnostics, it has greatly contributed to our knowledge of JD related management practices in Ireland and improves understanding of practical measures necessary to interpret ELISA diagnostics and aid JD control. It has identified numerous areas of future research that may ultimately lead to a decrease in MAP in Ireland thereby improving animal welfare and contributing to the competitiveness and efficiency of the Irish dairy industry.
Chapter 10: Publication List
10.1 Scientific Publications


10.2 Conference Proceedings


- Kennedy A, Byrne N, O’Mahony J, Sayers R. (2016). Comparative study investigating interference of SICCT on paratuberculosis ELISA in a herd


10.3 Popular Press Publications


Chapter 11: References


Behr, M. A. and D. M. Collins. 2010. Paratuberculosis: organism, disease, control. CABI.


avium subspecies paratuberculosis in 21 cows from herds affected by Johne's disease. Veterinary Record 162(5):147-152.


Click, R. E. 2011. Successful treatment of asymptomatic or clinically terminal bovine Mycobacterium avium subspecies paratuberculosis infection (Johne's disease) with the bacterium Dietzia used as a probiotic alone or in combination with dexamethasone: adaption to chronic human diarrheal diseases. Virulence 2(2):131-143.


207


Dillon, P. 2011. The Irish dairy industry – Planning for 2020

208


216


with two doses of Mycobacterium avium subspecies paratuberculosis using pathology and tissue culture. Veterinary research 44(1):94.


220


223


