Aus der Klinik für Wiederkäuer mit Ambulanz und Bestandsbetreuung

(Lehrstuhl für Innere Medizin und Chirurgie der Wiederkäuer: Prof. Dr. W. Klee)

der Ludwig-Maximilians-Universität München

und dem

Department of Population Medicine and Diagnostic Sciences,

College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

Arbeit angefertigt unter Leitung von

Ynte H. Schukken, DVM, PhD, Professor of Epidemiology and Herd Health

Survival of different *Mycobacterium avium* subsp. *paratuberculosis* strains in bovine monocyte-derived macrophages

Inaugural-Dissertation

zur Erlangung der tiermedizinischen Doktorwürde

der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

von

Nicole Severine Gollnick

aus Varel

München 2008

Gedruckt mit Genehmigung der Tierärztlichen Fakultät der

Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Braun
Berichterstatter:	Univ.-Prof. Dr. Klee
Korreferent:	Univ.-Prof. Dr. Kaspers

Tag der Promotion: 08. Februar 2008

Die Neugier steht immer an erster Stelle eines Problems, das gelöst werden will.
Galileo Galilei (1564-1642)

Meinen Eltern

Table of contents

1. Abbreviations

BT	Bluetongue disease
BHV-1	Bovine herpes virus-1
BVD	Bovine virus diarrhea
CFDA	Carboxyfluorescein diacetate
CFU	Colony forming units
CR	Complement receptor
DAPI	4', 6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxid
ELISA	Enzyme-linked immunosorbent assay
EMA	Ethidium monoazide
FACS	Fluorescence-activated cell sorting
FC	Fecal culture
FITC	Fluorescein isothiocyanate
FMD	Foot-and-mouth disease
FWS	Forward scatter
HEYM	Herrold's egg yolk medium
IBR	Infectious bovine rhinotracheitis
IL	Interleukin
INF	Interferon
MDMs	Monocyte-derived macrophages
MHC	Major histocompatibility complex
MLSSR	Multilocus short sequence repeat
MOI	Multiplicity of infection
NAHMS	National Animal Health Monitoring System
NLR	NOD-like receptor
NOD2	Nucleotide-binding oligomerization domain 2

NRAMP	Natural resistance-associated macrophage protein
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain-reaction
PRR	Pattern-recognition receptors
Slc11a1	Solute carrier family 11a member 1
SSC	Sideward scatter
SSR	Short sequence repeat
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-alpha
TRITC	Tetramethylrhodamine isothiocyanate

2. Introduction

Paratuberculosis, commonly known as Johne's disease, is caused by the intracellular bacterium *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*). The disease is characterized by a chronic and incurable granulomatous enteritis mainly affecting ruminants. Its pathogenesis is primarily determined by host immune reactions in attempt to eliminate the pathogen which persistently infects macrophages. Animals mostly get infected via the fecal-oral route during the first weeks of life usually followed by a long incubation period of 2-10 years. Currently available cost-effective diagnostic tools are not sufficient to identify the majority of animals in the preclinical phase making Johne's disease one of the most challenging infectious diseases of cattle. Spread of this worldwide occurring disease does not only affect the economy of the dairy industry but, due to its possible link to Crohn's disease, might potentially represent a threat to human health. To this day, our knowledge of host immune responses to *M. paratuberculosis* and the genetic constitution of the host potentially influencing infection as well as the genetic properties of the bacterium affecting its virulence remains incomplete.

It has been suggested that host genetics may play an important role in susceptibility to *M. paratuberculosis* infection and therefore are a possible reason for differences in the ability of macrophages to kill the organism between hosts of the same species. Furthermore, there have been indications that genotypic differences of *M. paratuberculosis* strains potentially influence the virulence of the pathogen. If true, breeding programs for ruminants as well as control strategies might have to be adapted in the future.

The presented doctoral study was performed to elucidate the influence of host infection status and strain differences on the outcome of *in vitro* infection in bovine monocyte-derived macrophages (MDMs). Objectives of this study were:

1) Evaluation of potential differences in phagocytic and killing ability of MDMs from cows which were test-positive by fecal culture and ELISA for *M. paratuberculosis* infection in comparison with matched cows of the same herd that had consistently shown negative results in both test categories.

2) Investigation of differences in intracellular survival in MDMs between different strains of *M. paratuberculosis*.

3) Determination of the cytotoxicity of the different *M. paratuberculosis* strains used in the experiment.

3. Literature review

3.1. History and general introduction

In the winter of 1894/95 the German veterinarian Friedrich Harms from Jever in Lower Saxony treated a cow with progressing emaciation and diarrhea on several occasions. Despite the medical treatment, the condition of the approximately six year old animal deteriorated over a period of six months, and the animal was finally culled on March 8, 1895. Harms, who suspected intestinal tuberculosis to be the underlying cause of the cow's condition, performed a necropsy on the animal. Uncertain of his diagnosis because his findings did not concur with the typical pathological picture of intestinal tuberculosis he sought a specialist opinion and sent sections of the pathologically altered jejunum, caecum and omentum to the veterinary pathology institute in Dresden for further investigations. The case was handled by Dr. Johne, pathologist of the veterinary School in Dresden and his American colleague Dr. Frothingham, visiting from Boston, MA, USA. Based on macroscopic and histological findings both concluded that this was an unusual case of bovine tuberculosis potentially caused by an avian tubercle bacillus which they found to be worth publishing in the case report "Ein eigenthümlicher Fall von Tuberkulose beim Rind" (Johne and Frothingham, 1985). It became the first scientifically documented case of paratuberculosis, in the English speaking world now commonly known as Johne's disease. In 1906, Bang recognized that the disease, despite its resemblance with intestinal tuberculosis, was in fact not caused by tubercle bacilli. But it took another four years until Twort was able to isolate and cultivate the causative agent, today known as *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) (Bang, 1906; Chiodini et al., 1984; Thorel et al., 1990).

Johne's disease is a chronic granulomatous enteritis, regional lymphangitis and lymphadenitis of ruminants (Buergelt et al., 1978; Clarke, 1997). In cattle, clinical disease is characterized by chronic, progressive loss of body condition, and intermittent or persistent diarrhea with fecal shedding of the organism. The disease is untreatable and eventually results in death (Clarke, 1997). Infected animals pass through two preclinical stages of the disease during an incubation period of 2 to 10 years: During the first, silent stage, *M. paratuberculosis* exposure usually remains undetectable using the currently available tests, while animals in the second preclinical stage may have antibodies detectable by available serological tests and/or may shed the bacteria in their feces (Whitlock and Buergelt, 1996). Not all animals, even when exposed to large numbers of bacteria, become persistently infected (Coussens, 2001; Gilmour and Angus, 1976). Possibly, the genetic constitution of the host and different genotypic properties of the infectious agent determine the outcome of infection.

In over a century since its discovery, Johne's disease has fascinated and frustrated many scientists. A plethora of investigations on pathogenesis and transmission, host range evaluation, development of diagnostic tests, design of eradication and control programs including vaccination regimes and management strategies, studies of the etiologic agents biology and the immune reactions of the host, have been conducted but still leave many questions yet to be answered (Barksdale and Kim, 1977; Buergelt et al., 1978; Chiodini et al., 1984; Clarke, 1997; Collins, 1996; Coussens, 2001; Harris and Barletta, 2001; Kennedy and Benedictus, 2001; Sweeney, 1996; Whitlock and Buergelt, 1996).

3.2. The infectious agent

Mycobacteria belong to the family *Mycobacteriaceae* with the only genus *Mycobacterium*. These aerobic, non-motile, rod-shaped, Gram-positive and acid-fast bacteria

can be subdivided into rapid- and slow-growing mycobacteria. The species *M. avium* belongs to the slow-growing group of mycobacteria and can be divided into four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *paratuberculosis (M. paratuberculosis)* and recently *M. avium* subsp. *hominissuis* (Mijs et al., 2002; Thorel et al., 1990). Besides *M. avium* subsp. *avium* and *M. paratuberculosis* the group of slow-growing mycobacteria comprises other important pathogens such as *M. tuberculosis*, *M. leprae*, and *M. bovis* affecting humans and various animal species (Wayne and Kubica, 1986).

M. paratuberculosis is a small (0.5 x 1.5 µm) rod-shaped, Gram-positive, acid-fast bacterium of high tenacity. *M. paratuberculosis* cannot multiply outside a host cell due to its dependency on mycobactin, an iron-chelating agent (Chiodini et al., 1984). The generation time in cell culture is 1.3 to 4.4 days (Lambrecht et al., 1988). Its cell wall, like in other bacteria of the genus mycobacteria, is composed of a thick waxy mixture of lipids and polysaccharides and is characterized by a high content of mycolic acid (Barksdale and Kim, 1977).

3.3. Prevalence and significance

Johne's disease presents a threat to the dairy industry worldwide. Available prevalence data need to be interpreted with caution, because diagnosis of animals in the preclinical period of Johne's disease is difficult and imprecise. It is assumed that for every cow with clinical signs of Johne's disease 25 other animal are infected. Only 15% to 25% of these infected animals can be detected with the current diagnostic tests if tests are not repeated (Whitlock and Buergelt, 1996). Therefore, the true prevalence of disease might well exceed the current estimates. Consequently, calculations on economic losses based on these prevalence studies are approximations which may not reflect the true degree of economic damage. In the USA,

seroprevalence on the individual animal level is reported to be 2.5% with 40.6% of herds infected. These results are based on a survey on 967 dairy herds which did not vaccinate against Johne's disease with 31745 animals in 20 states of the USA (NAHMS, 1997). Currently, there are no data available on the prevalence of Johne's disease in Germany. By estimate only, 5-15% of German dairy herds are suspected to be affected by the disease (Stratmann et al., 2005).

Significant milk production losses and lower slaughter weight redound directly to loss of income while increase in premature culling leads to higher costs for replacements. Reduction in reproductive performance and higher incidence of mastitis, both clinical and subclinical, are suspected, but a consistent connection with Johne's disease has yet to be shown (Harris and Barletta, 2001; Johnson-Ifearulundu et al., 1999; McKenna et al., 2006; Wells et al., 1998). Based on the National Animal Health Monitoring System study "Johne's Disease on US Dairy Operations" (NAHMS, 1997), Ott et al. (1999) reported that the average annual losses to the US dairy industry caused by reduction in productivity as a result of Johne's disease infections amount up to 200 to 250 million USD.

3.4. Host range

Not only cattle but all ruminant species are susceptible to paratuberculosis (Chiodini et al., 1984); and in recent years *M. paratuberculosis* infections associated with clinical signs of paratuberculosis have also been reported in lagomorphs (European brown hares, wild rabbits), rodents (woodmice, rats), carnivores (stoats, weasels, badgers, foxes) and corvids (jackdaws, rooks, crows) (as reviewed by Daniels et al., 2003). The role of paratuberculosis-infected non-ruminants in propagation of *M. paratuberculosis* in the bovine population has not yet been

well evaluated but it can be suspected that interspecies transmission might play an important role in the epidemiology of the disease.

3.5. Zoonotic potential

For many years the zoonotic potential of Johne's disease has been discussed very controversially. Already Dalziel (1913) suggested a connection between Johne's disease and a human ailment of similar pathological and clinical findings, which later became known as Crohn's disease. But to this day, scientists have not been able to clearly prove the etiological role of *M. paratuberculosis* in Crohn's disease, and further investigations are needed (Badiola et al., 2000; Behr and Schurr, 2006; Chamberlin and Naser, 2006). If in fact the association between the two diseases was verified and Johne's disease became a serious public health concern the impact on the cattle industry could be devastating (Greger, 2001; Shulaw et al., 2003; Stabel, 2000b).

3.6. Pathogenesis

3.6.1. Infection

Animals usually become infected through oral uptake of the organism from contaminated feed or environment (Sweeney, 1996) and potentially via infected macrophages present in colostrum and milk (Streeter et al., 1995). *In utero* infections are possible in dams in advanced stages of the disease (Sweeney et al., 1992). Calves up to the age of six months are more susceptible to infection with *M. paratuberculosis* than older animals (Payne and Rankin, 1961; Taylor, 1953). Ingested bacteria are predominantly taken up by membranous epithelial (M) cells covering the ileal Peyer's patches which present them to unactivated

macrophages beneath their basal membranes (Momotani et al., 1988). In ruminants, changes of the constitution of mucosal lymphoid tissues take place during the first months of life (Tizard, 2004; Yasuda et al., 2006). In lambs the ileal Peyer's patches begin to decrease in size after three months and regress entirely until adulthood (Reynolds and Morris, 1983). These changes might be of relevance to the increase in resistance to infection with *M. paratuberculosis*. Macrophages, large phagocytic leukocytes, belong to the innate immune system. They ingest presented antigens such as bacteria and usually destroy them efficiently through the generation of nitric oxide (NO), leading to the production of highly reactive and toxic oxidants (Tizard, 2004). Based on an *in vitro* study by Zhao et al. (1997) there is, however, evidence that the amount of NO produced by bovine macrophages is insufficient for killing intracellular *M. paratuberculosis*. Macrophages possess pattern-recognition receptors (PRRs) which recognize highly conserved molecules or molecular patterns called pathogen-associated molecular patterns (PAMPs) such as glycolipid of mycobacteria. PRRs can be integrated into the cell membrane or can be found inside the cells (Akira et al., 2006; Tizard, 2004). A number of cell surface PRRs are associated with the uptake of mycobacteria species by macrophages: Toll-like receptors TLR2 and TLR4, complement receptors CR1, CR3 and CR 4, fibronectin receptors, and mannose receptors (Bermudez et al., 1991; Danelishvili et al., 2007; Jo et al., 2007). Additionally the nucleotide-binding oligomerization domain 2 (NOD2) belonging to the NOD-like receptor (NLR) family has been identified as an important intracellular PRR in mycobacterial infections (Ferwerda et al., 2007). Mycobacteria spp. have the ability to manipulate the function of macrophages and thus impede their own destruction. Interference with the formation of phagolysosomes plays an important role in the survival of Mycobacteria spp. inside host cells. The mechanisms that allow these bacteria to intervene with the immune cell function are complex and thus far only partly understood (Russell et al., 2002).

3.6.2. Preclinical stages of Johne's disease

Upon experimental infection, *M. paratuberculosis* can be found in corresponding lymph nodes of affected intestinal sections as early as one hour after inoculation (Wu et al., 2007a). In this early stage of the infection proinflammatory cytokines such as interleukin-1 (IL-1) and IL-12 are released by infected macrophages leading to recruitment of macrophages and lymphocytes to the site of infection. Further, $CD4^+$ T cells, cytolytic $CD8^+$ T cells and γδ T cells become activated by these proinflammatory cytokines. These cells in return communicate via direct cell-to-cell contact or through the cytokines interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) with infected and naïve macrophages. INF-γ plays a central role in controlling *M. paratuberculosis* infections. Its function is highly dependent on its local concentration. INF-γ activates naïve macrophages which are allured to sites of infection by various cytokines. Activated macrophages can destroy *M. paratuberculosis* and therefore help fight off new infections. Furthermore, INF-γ suppresses mycobacterial growth in persistently infected macrophages which also confines the damage induced by *M. paratuberculosis* infection. TNF-α is highly significant for the formation of granulomata that enclose persistently infected bacteria; it does not prevent the intracellular multiplication of *M. paratuberculosis*. Thus, the T-cell mediated immune response is not sufficient to eliminate persistently infected macrophages but may limit further infection of immune cells (reviewed by Coussens, 2001). During this silent stage of Johne's disease macroscopic alterations of affected tissue are rare and are usually seen in animals which have been experimentally infected (Clarke, 1997). Histologically, however, microgranulomata in the intestine and the lymph nodes may be found (Whitlock and Buergelt, 1996; Wu et al., 2007a). Due to the lack of sufficient antibody formation and absence of fecal shedding of the infectious agent,

animals in the silent stage of Johne's disease can usually only be detected by mesenteric lymph node biopsy (Pemberton, 1979).

In the second stage of the incubation period, generation of intestinal lesions progresses as a result of the host immune reaction in an attempt to eliminate the pathogen (Coussens, 2001). Adduction of vast numbers of macrophages and lymphocytes leads to thickening of the intestine and its mucosal surfaces becomes corrugated and granular in appearance. Lymphatic vessels of the mesentery may thicken and dilate. As a result of immune cell recruitment affected lymph nodes become enlarged and pale in color (Buergelt et al., 1978). When the cell-mediated response attenuates, the humoral immune response becomes dominant and the animal is no longer able to contain the infection (Coussens, 2001). Unlike for other diseases, antibodies do not provide protection against *M. paratuberculosis*. On the contrary, detection of antibodies to *M. paratuberculosis* heralds the clinical stage of paratuberculosis. The underlying causes for the failure of the cell-mediated immunity still remain unidentified (Koets et al., 1999; Stabel, 2000c). Towards the end of the preclinical phase bacteria may be detected in the feces by culture or polymerase chain reaction (PCR) (Collins, 1996).

3.6.3. Clinical stage

When the cell-mediated immune response abates bacteria spread to other intestinal and lymphatic sites as well as other organs such as the liver, kidney and the lung. As a result progressing damage of the intestines and other tissues occurs (Clarke, 1997). At this stage of disease elevated antibody titers and large numbers of bacteria in the feces can usually be detected by routine diagnostic tests. The first clinical sign of Johne's disease is the gradual weight loss despite a normal or increased appetite. Subsequently the manure consistency becomes more fluid. At first the diarrhea may be intermittend. Thirst of animals is usually

increased while vital signs remain normal (Whitlock and Buergelt, 1996). Later, animals become debilitated and emacicated. Due to the intestinal damage animals develop hypoproteinemia which can lead to edema especially in the submandibular region (bottle jaw). In advanced Johne's disease, when animals have lost a marked amount of weight, the usual fat layers surrounding the kidney, heart and even in the bone marrow may be missing completely. If animals have not been removed from the herd thus far they eventually become cachectic, anemic and too weak to rise before they die (Chiodini et al., 1984; Manning and Collins, 2001).

3.7. Diagnostics

Two categories of tests for Johne's disease exist: those that determine the pathogen itself and those that detect immune responses to *M. paratuberculosis*. Antigen can be demonstrated by acid-fast staining of fecal smears, bacteriological culture of fecal and tissue samples and PCR. Tests investigating the host immune response against *M. paratuberculosis* detect antibodies (Serological tests), delayed-type hypersensitivity reactions (Johnin test) or increased INF-γ levels (INF-γ ELISA). A number of highly specific and cost-effective tests is commercially available for detection of *M. paratuberculosis* infections. Tests which either detect the bacterium itself or antibodies against *M. paratuberculosis* are highly specific but, unfortunately, due to the biology of Johne's disease, the sensitivity of these tests depends mainly on the stage of the disease. For example, most animals begin shedding *M. paratuberculosis* in their feces before antibodies can be detected by ELISA (Sweeney et al., 2006). Sensitivity of these ELISA tests is higher in animals shedding large numbers of bacteria (heavy shedders) compared to low shedders (Whitlock et al., 2000). Thus, choosing the right test(s) in a given situation requires good knowledge of the particularities of available

tests as well as the pathogenesis of Johne's disease. For further details on diagnostic procedures for paratuberculosis the reader is referred to publications by Collins (1996), Harris and Barletta (2001), Homuth (2002) and Rideout et al. (2003).

3.8. Disease awareness and control

Since Johne's disease was first described in 1895 its spread throughout the world has been reported. With an exception of Sweden and certain states of Australia, it has become a common disease in all countries with a significant dairy industry (Chiodini, 1993; Kennedy and Allworth, 2000; Viske et al., 1996). Despite the growing economical impact of Johne's disease and the increase of concern over human health implications the awareness among dairy farmers is alarmingly low. According to the 1996 NAHMS study "Johne's disease on US Dairy Operations" in which 2,542 producers in the United States participated, only 18% of farmers considered themselves fairly knowledgeable of Johne's disease. 10% had never heard of it, 35% at least knew the name of the disease, while 37% stated to have a basic knowledge (NAHMS, 1997). Comparable data on the situation in Europe are not available but are likely to reflect the circumstances in North America.

Reasons for low disease awareness are multifarious: Nature of the disease, lack of producer education with regard to Johne's disease, a general low awareness for the importance of biosecurity measures and the absence of mandatory control programs in most countries.

Cattle owners usually do not perceive paratuberculosis as a problem. Clinical signs normally occur only after a very long incubation period and are not pathognomonic. If animals stay in the herd until the late preclinical or early clinical phase, other impairments of health, such as failure of reproductive performance, mastitis or lameness may be predominant

and the more obvious sign of the animal's health problem. As a result, animals often leave the herd without being diagnosed as Johne's disease-positive.

In the NAHMS study "Reference of 1996 Dairy Management Practices, Part 1" it was reported that 78% of farmers listed veterinarians as a very important source for information in regard to making health care decisions on dairy farms (NAHMS, 1996). Thus, insufficient education of cattle owners, especially by veterinarians, may be another reason for lack of Johne's disease awareness.

The most important factor for propagation of Johne's disease is the introduction of infected but clinically healthy appearing animals to a herd (Sweeney, 1996). Yet disease prevention by strict application of biosecurity measures is still not a well adapted concept on North American dairy farms and a similar situation can be assumed for the European cattle industry. In a study with 2,194 participants 39% of cattle owners stated that they had brought at least one new animal onto their farm premises during 2006. Only 20% of the new arrivals were quarantined and less that 50% of producers required the purchased animals to be vaccinated against infectious diseases such as bovine virus diarrhea (BVD), infectious bovine rhinotracheitis (IBR) or leptospirosis prior to arrival on their farm (NAHMS, 2007). But these data only illustrate the overall attitude of producers concerning disease prevention. The situation for Johne's disease is even more complicated as many animals infected with *M. paratuberculosis* would not even be identifiable by commonly available tests.

Paratuberculosis sanitation programs of most countries, if they exist, are voluntary and procedures vary greatly from country to country. For instance in Germany, there is still lack of a national approach to Johne's eradication (Klee et al., 2002). It has been demonstrated in the past that success of control and eradication programs for animal diseases are correlated to the factors motivating producers to participate. Obligatory programs by national or international

law or penalty and incentive based programs initiated by the food processing industry have proven successful. Elimination of foot-and-mouth disease (FMD), bovine tuberculosis and brucellosis in Germany or the reduction of mastitis incidence in many industrialized countries are just a few examples (Valeeva et al., 2007). In case of Johne's disease, national eradication programs in Australia and Sweden have demonstrated the success of strategies in paratuberculosis control (Kennedy and Allworth, 2000; Viske et al., 1996).

German farmers are currently greatly affected by legal measures focusing on eradication of diseases such as bovine herpes virus-1 (BHV-1) infections, BVD and at present bluetongue disease (BT). Restrictions of international and national trade in regard to these diseases emphasize the economic importance of measures that have to be taken. Furthermore, unlike in Johne's disease, clinical signs for IBR, BVD and BT can be dramatic which facilitates communication of the necessity of reaching a disease-free status. Johne's disease, however, is thus far not subject to international economic sanctions (Rideout et al., 2003). Control of the disease, especially the attempt to eradicate the pathogen from a herd, demands exceptional management skills, good knowledge of etiology and pathogenesis of paratuberculosis, and patience. Reaching a disease-free status of an infected herd is labor-intensive and costly. It necessitates strict application of hygienic measures, involves culling of animals that might still appear healthy and highly productive, requires regular testing and demands for closing-off the herd. Usually the lack of financial benefits keeps producers that are aware of Johne's disease from participating in elaborate voluntary eradication programs (van Roermund et al., 2005). Some cattle owners might even feel that not knowing the current paratuberculosis status of their herd may be preferable (Klee et al., 2002).

While eradication of Johne's disease might not be possible with the current state of science and with presently available resources (Chiodini, 2005), control of *M.*

paratuberculosis infections could, without doubt, be improved by introduction of respective legal guidelines and preferably international standards for implementation of control programs and further education of veterinarians and cattle owners. But unless Johne's disease will be confirmed as a zoonosis, necessary legal actions by state executives are not to be expected in the near future. Nonetheless, filling the gaps which remain in the understanding of pathogenesis, host immune responses, bacterial structure and epidemiology of paratuberculosis will increase the likelihood of success for future control and potentially eradication programs.

3.9. Genetic susceptibility

For almost a century genetics has provided a major preoccupation for scientists in infectious disease susceptibility. A range of clinical phenotypes associated with infectious disease, racial differences in susceptibility, and twin studies all redounded to the view that the host genotype contributes to disease severity (Blackwell, 2001). A number of single gene defects in humans and mice have been identified which determine susceptibility to mycobacteria and other intracellular pathogens. Especially the solute carrier family 11a member 1 (Slc11a1) gene (formerly NRAMP1 gene) coding for a proton/divalent cation transporter located in the phagolysosomes membrane of macrophages has been studied in regard to numerous infectious and autoimmune diseases, including mycobacterial infections (Blackwell et al., 2001; Eichner Techau et al., 2007; Levin and Newport, 2000). Also defects in other genes critical for macrophage up-regulation, including IFN-γ, IL-12, the IFN-γ receptor, the TNF receptor, and the IL-12 receptor, are associated with decreased resistance to mycobacterial infection in humans (Levin and Newport, 2000). In recent years evidence for inherited predisposition to *M. paratuberculosis* infections has increased (Gonda et al., 2006;

Koets et al., 2000; Mortensen et al., 2004; Nielsen et al., 2002), yet potential genetic factors influencing resistance to Johne's disease are still unidentified. The bovine equivalent of the Slc11a1 gene has been identified, and was linked to resistance of cattle to *Brucella abortus* infections (Adams and Templeton, 1998; Barthel et al., 2001; Feng et al., 1996). A connection between polymorphisms of the Slc11a1 gene and resistance or susceptibility to mycobacterial infections in cattle has thus far not been demonstrated (Barthel et al., 2000). Reddacliff et al. (2005), however, found an association between host MHC-II and NRAMP alleles with *M. paratuberculosis* clinical status in sheep. Though further research is necessary, a potential future approach in Johne's disease control could be the reduction of *M. paratuberculosis* infections through selection of genetically resistant animals (Koets et al., 2000).

3.10. Molecular strain typing

Since the polymerase chain reaction (PCR) technique was invented by Mullis in 1983 it has revolutionized the world of molecular biology and augmented the knowledge of Johne's disease during the past two decades (Mullis, 1990). Early epidemiological studies identified that Johne's disease in cattle and sheep is caused by different bacterial strains. Differentiation of these strains was based on phenotypic differences such as colony pigmentation and colony surface constitution. Growth rate of bacteria was also used for determination of strain type (Taylor, 1951). Nowadays genotypic differentiation of strains is possible by application of molecular methods which are mostly based on PCR. An excellent introduction to the different available techniques is provided through recent reviews by Motiwala et al. (2006) and Sohal et al. (2007). Source tracking is one of the most common applications for molecular epidemiologic methods (Zadoks and Schukken, 2006). Strain typing can elucidate from where the infectious agent originated and may help to understand ways and risk factors for

transmission. Strain typing also allows investigating the genomic diversity of *M. paratuberculosis* strains and may permit the identification of highly virulent strains. Identification of the origin of infection which could be an animal in the herd, animals of other species which share the same pasture (e.g. wild rabbits) or an environmental source as well as detection of an animal shedding bacteria of a highly virulent strain could help veterinarians and cattle owners to focus on more sophisticated control measures. Furthermore, differentiation of isolates could elucidate mechanisms of host-specificity and association of specific genotypes with clinical disease versus subclinical states (Motiwala et al., 2006).

3.11. References

Adams, L.G., Templeton, J.W., 1998, Genetic resistance to bacterial diseases of animals. Rev. Sci. Tech. 17, 200-219.

Akira, S., Uematsu, S., Takeuchi, O., 2006, Pathogen recognition and innate immunity. Cell 124, 783-801.

Badiola, J.J., Bakker, D., Garcia Marin, J.F., Gilot, P., Hermon-Taylor, J., Sharp, J.M., Shivananda, S., Thorel, M.F., Vuitton, D. 2000. Possible links between Crohn's disease and Paratuberculosis (European Commission, Directorate - General Health and Consumer Protection, SANCO/B3/R16/2000), p. 76.

Bang, B., 1906, Chronische pseudotuberkuloese Darmentzuendung beim Rinde. Berliner Tierärztliche Wochenschrift 42, 49-57.

Barksdale, L., Kim, K.S., 1977, Mycobacterium. Bacteriol. Rev. 41, 217-372.

Barthel, R., Feng, J., Piedrahita, J.A., McMurray, D.N., Templeton, J.W., Adams, L.G., 2001, Stable transfection of the bovine NRAMP1 gene into murine RAW264.7 cells: effect on Brucella abortus survival. Infect. Immun. 69, 3110-3119.

Barthel, R., Piedrahita, J.A., McMurray, D.N., Payeur, J., Baca, D., Suarez, G.F., Perumaalla, V.S., Ficht, T.A., Templeton, J.W., Adams, L.G., 2000, Pathologic findings and association of Mycobacterium bovis infection with the bovine NRAMP1 gene in cattle from herds with naturally occurring tuberculosis. Am. J. Vet. Res. 61, 1140-1144.

Behr, M.A., Schurr, E., 2006, Mycobacteria in Crohn's disease: a persistent hypothesis. Inflamm. Bowel Dis. 12, 1000-1004.

Bermudez, L.E., Young, L.S., Enkel, H., 1991, Interaction of Mycobacterium avium complex with human macrophages: roles of membrane receptors and serum proteins. Infect. Immun. 59, 1697-1702.

Blackwell, J.M., 2001, Genetics and genomics in infectious disease susceptibility. Trends Mol. Med. 7, 521-526.

Blackwell, J.M., Goswami, T., Evans, C.A., Sibthorpe, D., Papo, N., White, J.K., Searle, S., Miller, E.N., Peacock, C.S., Mohammed, H., Ibrahim, M., 2001, SLC11A1 (formerly NRAMP1) and disease resistance. Cell Microbiol. 3, 773-784.

Buergelt, C.D., Hall, C., McEntee, K., Duncan, J.R., 1978, Pathological evaluation of paratuberculosis in naturally infected cattle. Vet. Pathol. 15, 196-207.

Chamberlin, W.M., Naser, S.A., 2006, Integrating theories of the etiology of Crohn's disease. On the etiology of Crohn's disease: questioning the hypotheses. Med. Sci. Monit. 12, RA27-33.

Chiodini, R.J., 1993, The History of Paratuberculosis (Johne's disease). A Review of the Literature 1895-1992. International Association for Paratuberculosis, 658 p.

Chiodini, R.J., 2005. The History of Paratuberculosis. In: Proceedings of Eighth International Colloquium on Paratuberculosis, Copenhagen, Denmark, pp. 1-4.

Chiodini, R.J., Van Kruiningen, H.J., Merkal, R.S., 1984, Ruminant paratuberculosis (Johne's disease): the current status and future prospects. Cornell Vet. 74, 218-262.

Clarke, C.J., 1997, The pathology and pathogenesis of paratuberculosis in ruminants and other species. J. Comp. Pathol. 116, 217-261.

Collins, M.T., 1996, Diagnosis of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 12, 357-371.

Coussens, P.M., 2001, Mycobacterium paratuberculosis and the bovine immune system. Anim. Health Res. Rev. 2, 141-161.

Dalziel, T.K., 1913, Chronic Interstitial Enteritis. British Medical Journal, 1058-1070.

Danelishvili, L., Wu, M., Stang, B., Harriff, M., Cirillo, S., Cirillo, J., Bildfell, R., Arbogast, B., Bermudez, L.E., 2007, Identification of Mycobacterium avium pathogenicity island important for macrophage and amoeba infection. Proc. Natl. Acad. Sci. U.S.A. 104, 11038-11043.

Daniels, M.J., Hutchings, M.R., Beard, P.M., Henderson, D., Greig, A., Stevenson, K., Sharp, J.M., 2003, Do non-ruminant wildlife pose a risk of paratuberculosis to domestic livestock and vice versa in Scotland? J. Wildl. Dis. 39, 10-15.

Eichner Techau, M., Valdez-Taubas, J., Popoff, J.F., Francis, R., Seaman, M., Blackwell, J.M., 2007, Evolution of differences in transport function in SLC11A family members. J. Biol. Chem. [epub ahead of print] published on October 10, 2007 as manuscript M707057200.

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

Ferwerda, G., Kullberg, B.J., de Jong, D.J., Girardin, S.E., Langenberg, D.M., van Crevel, R., Ottenhoff, T.H., Van der Meer, J.W., Netea, M.G., 2007, Mycobacterium paratuberculosis is recognized by Toll-like receptors and NOD2. J. Leukoc. Biol. 82, 1011-1018.

Gilmour, N.J., Angus, K.W., 1976, The specificity and sensitivity of the fluorescent antibody test in cattle experimentally infected with Mycobacterium avium and Mycobacterium johnei. Res. Vet. Sci. 20, 6-9.

Gonda, M.G., Chang, Y.M., Shook, G.E., Collins, M.T., Kirkpatrick, B.W., 2006, Genetic variation of Mycobacterium avium ssp. paratuberculosis infection in US Holsteins. J. Dairy Sci. 89, 1804-1812.

Greger, M. 2001. Paratuberculosis and Crohn's Disease: Got Milk? http://www.mad-cow.org/00/paraTB.html (last access: 09/01/07).

Harris, N.B., Barletta, R.G., 2001, Mycobacterium avium subsp. paratuberculosis in Veterinary Medicine. Clin. Microbiol. Rev. 14, 489-512.

Homuth, M., 2002, Diagnostik der Paratuberkulose. Dtsch. Tierärztl. Wochenschr. 109, 516-519.

Jo, E.K., Yang, C.S., Choi, C.H., Harding, C.V., 2007, Intracellular signalling cascades regulating innate immune responses to Mycobacteria: branching out from Toll-like receptors. Cell Microbiol. 9, 1087-1098.

Johne, A., Frothingham, L., 1985, Ein eigenthuemlicher Fall von Tuberculose beim Rind. Dtsch. Z. Thiermed. Vergl. Path. 21, 438-454.

Johnson-Ifearulundu, Y., Kaneene, J.B., Lloyd, J.W., 1999, Herd-level economic analysis of the impact of paratuberculosis on dairy herds. J. Am. Vet. Med. Assoc. 214, 822-825.

Kennedy, D.J., Allworth, M.B., 2000, Progress in national control and assurance programs for bovine Johne's disease in Australia. Vet. Microbiol. 77, 443-451.

Kennedy, D.J., Benedictus, G., 2001, Control of Mycobacterium avium subsp. paratuberculosis infection in agricultural species. Rev. Sci. Tech. 20, 151-179.

Klee, W., Baumgartner, W., Stürner, M., 2002, Forensische Aspekte bei der Paratuberkulose des Rindes. Deutsches Tierärzteblatt 10, 1048-1049.

Koets, A., Rutten, V.P.M.G., Hoek, A., Bakker, D., van, Z.F., Muller, K., van, E.W., 1999. The T(h)1 to T(h)2 Shift in Bovine Paratuberculosis and the Role of Apoptotic Cell Death. In: Proceedings of Sixth International Colloquium on Paratuberculosis, Melbourne, Australia, 1999, pp. 585-592.

Koets, A.P., Adugna, G., Janss, L.L., van Weering, H.J., Kalis, C.H., Wentink, G.H., Rutten, V.P., Schukken, Y.H., 2000, Genetic variation of susceptibility to Mycobacterium avium subsp. paratuberculosis infection in dairy cattle. J. Dairy Sci. 83, 2702-2708.

Lambrecht, R.S., Carriere, J.F., Collins, M.T., 1988, A model for analyzing growth kinetics of a slowly growing Mycobacterium sp. Appl. Environ. Microbiol. 54, 910-916.

Levin, M., Newport, M., 2000, Inherited predisposition to mycobacterial infection: historical considerations. Microbes Infect. 2, 1549-1552.

Manning, E.J., Collins, M.T., 2001, Mycobacterium avium subsp. paratuberculosis: pathogen, pathogenesis and diagnosis. Rev. Sci. Tech. 20, 133-150.

McKenna, S.L., Keefe, G.P., Tiwari, A., VanLeeuwen, J., Barkema, H.W., 2006, Johne's disease in Canada part II: disease impacts, risk factors, and control programs for dairy producers. Can. Vet. J. 47, 1089-1099.

Mijs, W., de Haas, P., Rossau, R., Van der Laan, T., Rigouts, L., Portaels, F., van Soolingen, D., 2002, Molecular evidence to support a proposal to reserve the designation

Mycobacterium avium subsp. avium for bird-type isolates and 'M. avium subsp. hominissuis' for the human/porcine type of M. avium. Int. J. Syst. Evol. Microbiol. 52, 1505-1518.

Momotani, E., Whipple, D.L., Thiermann, A.B., Cheville, N.F., 1988, Role of M cells and macrophages in the entrance of Mycobacterium paratuberculosis into domes of ileal Peyer's patches in calves. Vet. Pathol. 25, 131-137.

Mortensen, H., Nielsen, S.S., Berg, P., 2004, Genetic variation and heritability of the antibody response to Mycobacterium avium subspecies paratuberculosis in Danish Holstein cows. J. Dairy Sci. 87, 2108-2113.

Motiwala, A.S., Li, L., Kapur, V., Sreevatsan, S., 2006, Current understanding of the genetic diversity of Mycobacterium avium subsp. paratuberculosis. Microbes Infect. 8, 1406-1418.

Mullis, K.B., 1990, The unusual origin of the polymerase chain reaction. Sci. Am. 262, 56-61, 64-55.

NAHMS 1996. Part I: Reference of 1996 Dairy Management Practices. USDA:APHIS:VS, CEAH, National Animal Health Monitoring System, Fort Collins, CO.

NAHMS 1997. Johne's Disease on US Dairy Operations. USDA:APHIS:VS, CEAH, National Animal Health Monitoring System. Fort Collins, CO.

NAHMS 2007. Part I: Reference of Dairy Cattle Health and Management Practices in the United States, 2007. USDA:APHIS:VS, CEAH, National Animal Health Monitoring System, Fort Collins, CO.

Nielsen, S.S., Grohn, Y.T., Quaas, R.L., Agger, J.F., 2002, Paratuberculosis in dairy cattle: variation of the antibody response in offspring attributable to the dam. J. Dairy Sci. 85, 406-412.

Ott, S.L., Wells, S.J., Wagner, B.A., 1999, Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. 40, 179-192.

Payne, J.M., Rankin, J.D., 1961, A Comparison of the Pathogenesis of Experimental Johne's Disease in Calves and Cows. Res. Vet. Sci. 2, 175-179.

Pemberton, D.H., 1979, Diagnosis of Johne's disease in cattle using mesenteric lymph node biopsy: accuracy in clinical suspects. Aust. Vet. J. 55, 217-219.

Reddacliff, L.A., Beh, K., McGregor, H., Whittington, R.J., 2005, A preliminary study of possible genetic influences on the susceptibility of sheep to Johne's disease. Aust. Vet. J. 83, 435-441.

Reynolds, J.D., Morris, B., 1983, The evolution and involution of Peyer's patches in fetal and postnatal sheep. Eur. J. Immunol. 13, 627-635.

Rideout, B.A., Brown, S.T., Davis, W.C., Gay, J.M., Gianella, R.A., Hines, M.E., Hueston, W.E., Hutchinson, L.J., 2003, Diagnosis and Control of Johne's Disease. Committee on Diagnosis and Control of Johne's Disease, National Research Council, 244 p.

Russell, D.G., Mwandumba, H.C., Rhoades, E.E., 2002, Mycobacterium and the coat of many lipids. J. Cell Biol. 158, 421-426.

Shulaw, W.P., Larew-Naugle, A., Torrence, M.E., Isaacson, R.E., 2003, Paratuberculosis: A Food Safety Concern?, In: Torrence, M.E., Isaacson, R.E. (Eds.) Microbial Food Safety in Animal Agriculture. Iowa State Press, Ames, IO.

Sohal, J.S., Singh, S.V., Subodh, S., Sheoran, N., Narayanasamy, K., Singh, P.K., Singh, A.V., Maitra, A., 2007, Mycobacterium avium subspecies paratuberculosis diagnosis and geno-typing: Genomic insights. Microbiol. Res. 2006; (2007), [epub ahead of print] doi:10.1016/j.micres.2007.1003.1005.

Stabel, J.R., 2000a, Johne's disease and milk: do consumers need to worry? J. Dairy Sci. 83, 1659-1663.

Stabel, J.R., 2000b, Transitions in immune responses to Mycobacterium paratuberculosis. Vet. Microbiol. 77, 465-473.

Stratmann, J., Homuth, M., Gerlach, G.F., 2005, Überlegungen zur Kontrolle und Bekämpfung der Paratuberkulose in Milchviehbeständen. Dtsch. Tierärztl. Wochenschr. 112, 304-306.

Streeter, R.N., Hoffsis, G.F., Bech-Nielsen, S., Shulaw, W.P., Rings, D.M., 1995, Isolation of Mycobacterium paratuberculosis from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. 56, 1322-1324.

Sweeney, R.W., 1996, Transmission of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 12, 305-312.

Sweeney, R.W., Whitlock, R.H., McAdams, S., Fyock, T., 2006, Longitudinal study of ELISA seroreactivity to Mycobacterium avium subsp. paratuberculosis in infected cattle and culture-negative herd mates. J. Vet. Diagn. Invest. 18, 2-6.

Sweeney, R.W., Whitlock, R.H., Rosenberger, A.E., 1992, Mycobacterium paratuberculosis isolated from fetuses of infected cows not manifesting signs of the disease. Am. J. Vet. Res. 53, 477-480.

Taylor, A.W., 1951, Varieties of Mycobacterium johnei isolated from sheep. J. Pathol. Bacteriol. 63, 333-336.

Taylor, A.W., 1953, Experimental Johne's disease in cattle. J. Comp. Pathol. 63, 355-367.

Thorel, M.F., Krichevsky, M., Levy-Frebault, V.V., 1990, Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of Mycobacterium avium, and description of Mycobacterium avium subsp. avium subsp. nov., Mycobacterium

avium subsp. paratuberculosis subsp. nov., and Mycobacterium avium subsp. silvaticum subsp. nov. Int. J. Syst. Bacteriol. 40, 254-260.

Tizard, I.R., 2004, Veterinary Immunology: An Introduction, 7th Edition Edition. Elsevier.

Valeeva, N.I., Lam, T.J., Hogeveen, H., 2007, Motivation of dairy farmers to improve mastitis management. J. Dairy Sci. 90, 4466-4477.

van Roermund, H.J.W., Weber, M.F., de Koeijer, A.A., Velthuis, A.G.J., de Jong, M.C.M., 2005. Development of a milk quality assurance program for paratuberculosis: from within- and between-herd dynamics to economic decision analysis. In: Proceedings of Eighth International Colloquium on Paratuberculosis, Copenhagen, Denmark, pp. 51-59.

Viske, D., Larsson, B., Engvall, A., Bölske, G., 1996. Paratuberculosis in Sweden. In: Proceedings of Fifth International Colloquium on Paratuberculosis, Madison, Wisconsin, USA.

Wayne, L.G., Kubica, G.P., 1986, Bergey's manual of systematic bacteriology, Vol 2, 1 Edition. Holt, J. G., Sneath, P. H. A., Mair, N. S., Sharpe, M. E., 1435-1457 pp.

Wells, S.J., Ott, S.L., Seitzinger, A.H., 1998, Key health issues for dairy cattle - new and old. J. Dairy Sci. 81, 3029-3035.

Whitlock, R.H., Buergelt, C., 1996, Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet. Clin. North Am. Food Anim. Pract. 12, 345-356.

Whitlock, R.H., Wells, S.J., Sweeney, R.W., Van Tiem, J., 2000, ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. Vet. Microbiol. 77, 387-398.

Wu, C.W., Livesey, M., Schmoller, S.K., Manning, E.J., Steinberg, H., Davis, W.C., Hamilton, M.J., Talaat, A.M., 2007, Invasion and persistence of Mycobacterium avium subsp.

paratuberculosis during early stages of Johne's disease in calves. Infect. Immun. 75, 2110-2119.

Yasuda, M., Jenne, C.N., Kennedy, L.J., Reynolds, J.D., 2006, The sheep and cattle Peyer's patch as a site of B-cell development. Vet. Res. 37, 401-415.

Zadoks, R.N., Schukken, Y.H., 2006, Use of molecular epidemiology in veterinary practice. Vet. Clin. North Am. Food Anim. Pract. 22, 229-261.

Zhao, B., Collins, M.T., Czuprynski, C.J., 1997, Effects of gamma interferon and nitric oxide on the interaction of Mycobacterium avium subsp. paratuberculosis with bovine monocytes. Infect. Immun. 65, 1761-1766.

4. Publication

In Veterinary Immunology and Immunopathology (2007),

accepted for publication 25 July 2007

Survival of *Mycobacterium avium* **subsp.** *paratuberculosis* **in bovine monocyte-derived macrophages is not affected by host infection status but depends on the infecting bacterial genotype**

Nicole S. Gollnick[a,*], Rebecca M. Mitchell[b], Martin Baumgart[c], Harish K. Janagama[d], Srinand Sreevatsan[d], Ynte H. Schukken[b]

[a] Clinic for Ruminants, University of Munich, Sonnenstrasse 16, 85764 Oberschleissheim, Germany, phone: +49-89-21 80 788 50, fax: +49-89-21 80 788 51, e-mail address: nicole.gollnick@med.vetmed.uni-muenchen.de

[b] Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

[c] Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

[d] Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, USA

* Corresponding author

4.1. Abstract

In this study we investigated the ability of different *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) strains to survive in bovine monocyte-derived macrophages (MDMs) of cows naturally infected with *M. paratuberculosis* and control cows. We tested the hypotheses that infection status of cows affects macrophage killing ability and that survival of *M. paratuberculosis* in macrophages is dependent on the strain. Peripheral blood mononuclear cells (PBMC) were obtained from Johne's disease-positive (n = 3) and age and stage of lactation matched Johne's disease-negative (n = 3) multiparious cows. Following differentiation, MDMs where challenged in vitro with 4 *M. paratuberculosis* strains of different host specificity (cattle, sheep). Two hours and 2, 4, and 7 days after infection, ingestion and intracellular survival of *M. paratuberculosis* strains were determined by fluorescence microscopy. There was no effect of the origin of MDMs (Johne's disease-positive or control animals) on phagocytosis, survival of bacteria, or macrophage survival. In contrast, important strain differences were observed. These findings suggest that some *M. paratuberculosis* strains interfere more successfully than others with the ability of macrophages to kill intracellular pathogens which may make it important to include strain typing when designing control programs.

4.2. Keywords

Cattle; Mycobacterium paratuberculosis; Macrophage; PBMC; Genetic susceptibility

4.3. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*M. paratuberculosis*) causes paratuberculosis (Johne's disease), a chronic, incurable granulomatous enteritis of ruminants (Buergelt et al., 1978; Chiodini et al., 1984). It is widely accepted that cattle usually become infected *in utero* or as calves through oral uptake of the organism from contaminated feed or the environment (Sweeney, 1996; Sweeney et al., 1992). Infected animals pass through two preclinical stages of the disease during an incubation period of 2 to 10 years. During the first, silent stage, *M. paratuberculosis* infection usually remains undetectable by currently available tests, while animals in the second preclinical stage may have increased antibodies detectable by available serological tests and/or may shed the bacteria in their feces (Whitlock and Buergelt, 1996).

To this day, our knowledge of host immune responses to *M. paratuberculosis* and the genetic constitution of the host potentially influencing infection as well as the genetic properties of the bacterium affecting its virulence remains incomplete (Coussens, 2001; Motiwala et al., 2006). To elucidate the interaction between *M. paratuberculosis* and the host immune system, a number of studies have investigated gene expression of PBMC from Johne's disease-positive and healthy control animals (Coussens et al., 2003; Coussens et al., 2004a; Coussens et al., 2005; Coussens et al., 2004b). Further, research has been carried out on the differences in cytokine profiles of PBMC and monocytes from *M. paratuberculosis*-infected animals and control animals (Khalifeh and Stabel, 2004; Stabel, 2000a; Weiss et al., 2005). However, with the exception of a study by Weiss et al. (2005), none of the investigations have sufficiently addressed survival of the bacterium as a function of infection status of the animal.

Genetic predisposition to intracellular infections of macrophages with *Brucella abortus, M. bovis BCG,* and *S. dublin* has been documented in cattle (Qureshi et al., 1996) and has been suggested for infections of sheep and cattle with *M. paratuberculosis* (Gonda et al., 2006; Nielsen et al., 2002; Reddacliff et al., 2005). These studies suggest that host genetics may play an important role in susceptibility to *M. paratuberculosis* infection and therefore are a possible reason for differences in the ability of macrophages to kill the organism between hosts of the same species.

Phenotypic strain differences between *M. paratuberculosis* isolates causing Johne's disease in cattle and sheep have been identified as early as the middle of the last century (Taylor, 1951). Only now have recent developments in the field of molecular biology and genomics provided the research community with tools that facilitate genotypic characterization of *M. paratuberculosis* isolates, representing a major advancement for epidemiological investigations of the disease (Dohmann et al., 2003; Harris et al., 2006; Motiwala et al., 2006). Multilocus short sequence repeat (MLSSR) typing has been used in genotypic analysis of *M. paratuberculosis* isolates derived from individual animals of a diverse range of hosts (Amonsin et al., 2004; Motiwala et al., 2004). This allows the analysis of virulence of different genotypes *in vivo* and *in vitro,* which will improve our understanding of the molecular pathogenesis of Johne's disease and may aid in the design of a better strategy for controlling the infection.

The objectives of this study were twofold. First, we evaluated potential differences in phagocytic and killing ability of monocyte-derived macrophages (MDMs) from cows that were test-positive by fecal culture and ELISA for *M. paratuberculosis* infection in comparison with matched cows of the same herd that had consistently shown negative results in both test categories. Second, we evaluated differences in intracellular survival in MDMs between

different strains of *M. paratuberculosis*. Further, we studied the cytotoxicity of the different *M. paratuberculosis* strains used in the experiment.

4.4. Materials and Methods

4.4.1. Animals

The animals used in this study were in their third lactation and part of a 340-dairy-cow operation in New York State. The infection status of the animals had been monitored by serum ELISA (ParaCheck; CSL/Biocor, Omaha, NE) every three months and by fecal culture testing at least once every six months during a period of 16 months prior to initiation of the experiments. Fecal culture testing was conducted by the Johne's Research Laboratory, New Bolton Center, University of Pennsylvania, Kennett Square, PA, as described by Crossley et al. (2005). Johne's disease test-positive cows (n = 3) had been fecal culture-positive with between 0.75 and 50 CFU/tube for a minimum of three consecutive tests; they had also been positive by serum ELISA in at least one test. These animals showed no clinical signs of Johne's disease. Johne's disease test-positive animals had exclusively tested positive for infection with one *M. paratuberculosis* genotype (strain 1180, strain genotype described below) used in this study. The control animals (n = 3) had shown negative responses in all tests during the monitoring period. Control cows were matched with test-positive cows by days in milk, parity, and pregnancy status. None of the animals had shown clinical signs of any disease and had not been treated systemically with antimicrobial agents for at least 30 days prior to blood collection. Characteristics of the 6 animals in this study are presented in Table 1. The results of ELISA and fecal culture for all animals are detailed in Table 2.

4.4.2. Blood collection, isolation of PBMC, and preparation of autologous serum

Approximately 800 ml of blood for the isolation of PBMC were obtained from the jugular vein of each cow using sterile blood collection bags J-520Q or J-520D with citrate phosphate dextrose adenine solution (CPDA-1) or acid citrate dextrose solution (ACD) as anticoagulant (Jorgensen Laboratories, Inc., Loveland, CO). Additionally, between 150 and 200 ml of blood for the preparation of autologous serum were collected from the jugular vein or tail vein into 10-ml tubes without anticoagulant (Vacutainer™, Becton Dickinson, Franklin Lakes, NJ). All blood samples were stored at approximately 15 °C for four hours during transport to the laboratory.

Blood for the isolation of PBMC was transferred under sterile conditions from the blood collection bags into sterile glass bottles and subsequently into 50-ml centrifuge tubes. The blood was centrifuged at 1000 x g and 22 °C for 10 min. Buffy coats were harvested using sterile 5-ml plastic pipettes after discarding most of the plasma. Two buffy coats were pooled and resuspended in PBS (without Ca^{2+} and Mg^{2+}); the total volume of this mixture was adjusted to 50 ml. Twenty-five milliliter of cell suspension each were then carefully layered over 25 ml of Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO) in fresh centrifuge tubes. Cells were centrifuged at 400 x g and 22 °C for 30 min to separate erythrocytes and polymorphonuclear cells from mononuclear cells. PBMC were harvested from the PBS-Histopaque® interface and washed three times with PBS at 4 °C and 200 x g for 10 min. The isolated mononuclear cells were resuspended in RPMI 1640 with Glutamax™ I and 25 mM HEPES (Invitrogen, Carlsbad, CA). Except for cell cultures derived from cows 1180 and 1184, the growth medium was supplemented with 0.25 µg of amphotericin B (Nitrogen, Carlsbad, CA) per ml. The cell density was adjusted to approximately 7 x 10^6 cells/ml, and autologous serum was slowly added to the cell suspension. The final serum concentration of

the growth medium was set at 12% as recommended by Campbell and Adams (1992). PBMC were incubated for 5 days at 37 °C and 5% CO_2 in 350-ml Teflon jars (Savillex, Minnetonka, MN) and 50-ml Teflon flasks (Nalgene Company, Rochester, NY) to allow monocytes in the unfractionated PBMC cultures to develop into macrophage precursor cells.

To obtain autologous serum, corresponding blood samples in collection tubes were allowed to clot for at least 6 hours. Subsequently, the tubes were centrifuged at 4 °C and 1800 x g for 25 min, and serum was transferred into 50-ml centrifuge tubes and then micron filtered using 0.22-µm Steriflip-GP Filter Units (Millipore, Billerica, MA). The serum was aliquoted and stored at -20 °C until use.

4.4.3. Cell culture conditions and infection

After the incubation period, cells in Teflon containers were resuspended by careful pipetting and centrifuged at 4 °C and 200 x g for 10 min in a fresh sterile tube. The cell pellets were resuspended in RPMI 1640 with Glutamax™ I and 25 mM HEPES, and 2% of autologous serum (complete medium). Except for cell cultures derived from cows 1180 and 1184, the growth medium was supplemented with 0.25 µg of amphotericin B per ml. Aliquots of cells were prepared in a 1:10 dilution in Trypan Blue, and a cell count was performed with an Olympus CK2 light microscope utilizing a hemocytometer. Macrophage precursor cells could easily be identified by their size and granularity, which allowed an overall estimation of the number of these cells per ml of cell suspension. Further aliquots of the cell culture were prepared for histological investigation by cytocentrifugation and subsequent staining of the cells on glass slides with Wright's stain. This procedure was completed to validate the above described method to determine the macrophage precursor cell fraction. The cell density was adjusted with growth medium to the correct seeding density of macrophage precursor cells.

The vast majority of PBMC were utilized for experimental purposes not reported here. PBMC for the described assay were seeded at a density of 1 x 10^5 macrophage precursor cells into wells of 24-well culture plates containing 12 mm-diameter glass cover slips. Cell cultures were challenged with bacteria at multiplicity of infection (MOI) of 5:1 the next day. To avoid removal of lymphocytes from the cultures, only part of the culture medium was replaced 3 hours, 2 days, and 4 days after infection. Then, 0.2 ml of medium was carefully removed from the top of the undisturbed wells of the 24-well plates and 0.3 ml of fresh complete medium added.

4.4.4. Bacterial strains

Four *M. paratuberculosis* strains and one non-*M. paratuberculosis*-positive control (strain 6043, member of the *Mycobacterium avium-intracellulare complex*) as well as a negative control (medium alone) were used for the experiments. *M. paratuberculosis* strain 1018 (short sequence repeat [SSR] fingerprint: 7G4GGT) which had been isolated from a fecal sample of an individual animal at a dairy herd in Ohio where multiple strains of *M. paratuberculosis* were present. *M. paratuberculosis* strain 7565 (SSR fingerprint: 15G3GGT) had been isolated from an intestinal tissue sample of a sheep with Johne's disease. This strain showed typical ovine strain characteristics in culture. *M. paratuberculosis* strain 1180 (homologous farm strain) (SSR fingerprint: L1:7G L8:6GGT) was isolated from animal 1180 enrolled in this study. *M. paratuberculosis* strain 1099 (SSR fingerprint: L1:7G L8:5GGT) was also isolated from a Johne's disease-positive animal of the study farm, but none of the study animals were culture-positive for this heterologous farm strain. All strains were genotyped by a modified multilocus short sequence repeat (MLSSR) described by Amonsin et

al. (2004). Strains 1018, 7565 and 6043 are further characterized for differences in macrophage interaction elsewhere (Janagama et al., 2006).

All cell-infection experiments in this study were performed with bacteria derived from serial passages and dilutions of the respective bacterial stock cultures. Seven to 14 days prior to cell infection bacteria were grown in Middlebrook 7H9 broth (Difco laboratories, Detroit, MI) supplemented with 10% oleic acid albumin dextrose catalase (OADC; Becton Dickinson Microbiology System, Sparks, MD) and 2 µg/ml of Mycobactin J (Allied Monitor Inc., Fayette, MO). Bacterial density was determined using a hemocytometer count. In brief, each culture was vortexed for 10 seconds and subsequently incubated with 1:1 volume of 4% buffered formalin for 15 min after which it was syringed 10 times with a 23-g needle to break up clumps of bacteria. Based on the average of two hemocytometer counts, sufficient medium containing live bacteria was syringed 10 times with a 23-g needle and then diluted with complete medium to produce an MOI of 5:1 for cell infections. Negative control PBMC cultures received complete medium with an average volume of Middlebrook 7H9 medium supplemented with 10% OADC and 2 µg/ml of Mycobactin J to control for the effect of Middlebrook 7H9 broth on cell cultures.

4.4.5. Fluorescence microscopy

Samples for fluorescent microscopy were collected four times: 2 hours and 2, 4, and 7 days after experimental infection. First, 100 mg carboxyfluorescein diacetate (CFDA) (Invitrogen, Carlsbad, CA) were dissolved in 1000 µl DMSO, and 220 µl of this master mix were diluted 1:25 in infection medium. Then, 150 µl of CFDA solution were added to each well of the 24-well plate after removal of the growth medium. Cells were incubated for 20 min at 37 °C. Thereafter, coverslips were transferred into new 24-well plates. Coverslips were

fixed with 0.5 ml of 4% formaldehyde each for a minimum of 15 min at 4 °C. MDMs on the coverslips were subsequently permeabilized with 100% methanol for 20 min at -20 °C. Methanol was removed and the cells were counterstained with 0.5% Evans Blue in PBS for 20 min in the dark at room temperature, after which the coverslips were washed twice with PBS. The coverslips were removed from the wells, washed in PBS, and mounted with ProLong® Gold antifade reagent with DAPI (Molecular Probes, Eugene, OR) on glass slides. To prevent the mounting medium from dehydration coverslips were sealed using clear nail polish. Slides were stored in the dark at 4 °C. Slides containing three coverslips each (triplicate set-up) were assessed at 400 x magnification with an Olympus System Microscope BX41 and the FITC filter (excitation [ex] 480 nm, band pass [BP] 40, emission [em] 535 nm, BP 50); TRITC filter (ex 545, BP 30, em 620 nm, BP 60), and DAPI filter (ex 365 nm, BP 10, em 460 nm, BP 50). Images were captured by a MicroFire™ camera, Model S99809 (Optronics, Goleta, CA) and analyzed with PictureFrame™, Version 2.1 software (Optronics, Goleta, CA). Five defined visual fields were captured per coverslip. TRITC (cytoplasm of MDMs) and FITC (viable bacteria) filter images were merged in PictureFrame™, and cells were evaluated on the screen (Fig. 1). Only MDMs that were located entirely within the boundaries of the picture were considered. MDMs were classified in five different categories: MDMs with zero, 1 to 10, 11 to 20, 21 to 50, and >50 bacteria per cell.

4.4.6. Flow cytometric analysis

PBMC cell phenotypes were assessed for each animal by single-color flow cytometric analysis on the day after isolation and on the day after completion of the incubation period in Teflon jars. The monoclonal antibodies employed in this study were directed against CD4 (CACT138A), CD8 (CACT80C), the γδ T cell receptor 1-N24 (GB21A), CD14 (MM61A)

and a yet unspecified surface molecule which is expressed on bovine B cells and is recognized by the antibody BAQ155A. All antibodies were obtained from VMRD, Pullman, WA. Before fixing with 2% formaldehyde and subsequent antibody staining, cells were treated with ethidium monoazide (EMA; Molecular Probes, Eugene, OR) according to a protocol by De Rosa (2004) to mark dead PBMC. Individual cell culture aliquots ($1x10^6$ cells/100 µl aliquot) were stained with unconjugated primary antibodies and a FITC conjugated secondary antibody. Briefly, samples were incubated for 15 min on ice with (concentration 15 µg/ml) of the primary antibody, washed with FACS buffer (PBS with 5% FBS and 0.02% sodium azide) and finally stained with a secondary FITC-conjugated antibody for 20 min at a concentration of 10 µg/ml (goat anti-mouse IgM+IgG+IgA (H+L); SouthernBiotech, Birmingham, AL). After a final washing step, cell pellets were resuspended in 500 µl of FACS buffer and analyzed on a LSR II cytometer (BD Biosciences, San Jose, CA). The data generated were analyzed for 30,000 EMA negative cell events. Analysis was performed with FlowJo software Version 4.6.2 (Tree Star Inc., San Carlos, CA).

4.4.7. Statistical analysis

Results of the three separate phagocytosis experiments with a pair of one test-positive and one test-negative animal were combined. A linear mixed model was used for analysis of data. The proportion of MDMs and the number of bacteria per infected MDM were calculated for each of 5 viewing fields in the 3 cover slips on a slide during 4 time periods with 5 strains and one control in a total of 6 cows (3 test-positive and 3 test-negative) for a total of 2160 observations. To estimate the average number of bacteria per infected cell, the number of cells per category (1 to 10, 11 to 20, 21 to 50, and > 50) were multiplied by 5, 15, 35, and 75, respectively. Means were calculated for each relevant subgroup. Standard error estimates of

the mean were obtained using the square root of the usual (binomial and normal) variance divided by the sample size. The comparison of test-positive versus test-negative animals and the comparison of strains were performed in generalized linear mixed models. The proportion of infected cells or the number of bacteria per infected MDM was the outcome variable, while the experiment number (1, 2, 3), test-positive versus test-negative animals, strain indicator, and potential interactions were the predictor variables. Correlation within viewing field and within cover slip was accounted for using hierarchical random effects. The phagocytic index (percent infected cells times bacteria per infected cell) was used to combine parameters for bacteria per infected cell and percent infected cells in the raw data set as described by Zurbrick and Czuprynski (1987).

Flow cytometric data were checked for outliers. The Wilcoxon rank sum non-parametric method was used to compare cell populations between test-positive and test-negative animals and between the results on day 1 and day 5.

Statistical Analysis System (SAS) Version 9.1 (SAS Institute Inc., Cary, NC) was used to analyze the dataset. We controlled for experiment number in the analysis of the results. Statistical significance was set at $p = 0.05$.

4.5. Results

4.5.1. Johne's disease status of cows did not influence macrophage infection and survival of bacteria

Fluorescence microscopy was applied to visualize phagocytosed bacteria and to calculate the phagocytic index (Fig. 1). At 2 days post-infection, the phagocytic index was highest for all strains except strain 1099, which peaked 4 days post-infection. The positive

control showed a consistently high phagocytic index in all experiments. Table 3 shows the results of the parameters for percent of infected cells and bacteria per infected cell for the cells derived from test-positive and test-negative animals. Results are presented for the four measurement times (2 hours and 2, 4, and 7 days after infection) and for the 5 strains used. Results of generalized linear models showed no evidence of significant differences between infection results in cells derived from test-positive compared to cells derived from test-negative animals ($p > 0.9$) for any of the 5 bacterial strains. Figure 2 shows the results of the individual cows with the phagocytic index at 2 days after experimental infection for the homologous farm strain 1180 and the bovine strain 1018. There was no significant difference between the test-positive and test-negative animals.

4.5.2. M. paratuberculosis genotype affected the percent of infected MDMs and the total numbers of phagocytosed bacteria per infected cell

Differences between strains were observed in both the percentage of infected MDMs and the number of bacteria per infected MDM. Consistently, the homologous and heterologous *M. paratuberculosis* strains 1180 and 1099 from the source farm were closest in growth pattern to the positive control strain 6043, whereas bovine strain 1018 was consistently the least successful in both percent of infected MDMs and bacteria per infected MDM. Results are presented in Table 4. The differences in the phagocytic index of the three bovine *M. paratuberculosis* strains are given in Fig. 3A. The source farm strains were significantly different (generalized linear mixed model result, $p < 0.05$) from strain 1018, and were not significantly different from one another. The ovine strain initially showed a very high number of bacteria per MDM, but subsequently was the only strain that showed a significant decline in bacteria per infected cell over time (Fig. 3B).

4.5.3. Number of MDMs per visual field continuously increased over time

The average number of MDMs per visual field for most strains and the negative control constantly increased over time ($p < 0.001$). Test-negative animals tended to have higher numbers of MDMs per visual field ($p < 0.03$) than test-positive cows. Among the infected MDMs, those infected with ovine strain 7565 showed a pattern similar to that of the MDMs in non-infected wells, with a rapid increase between four and seven days. In contrast, MDMs infected with the bovine strains and the positive control strain 6043 did not show marked proliferation at measurement time 4 (Fig. 4).

4.5.4. Unfractionated PBMC flow cytometry results changed after 5 days of incubation

First, EMA-positive cells were gated to exclude dead cells. Remaining cells were further gated in the forward scatter (FSC) and sideward scatter (SSC) to exclude cell debris. Finally, specific antibody staining of the preselected cells was analyzed in the green fluorescence channel. Final results are presented in Fig. 5, which shows the cell profile separately for test-positive and test-negative animals on either day 1 or day 5 of PBMC culture. Non-parametric statistical testing indicated that the proportion of cells did not differ between test-positive and test-negative animals, but that there was a significantly different profile on day 5 compared to the starting distribution on day 1. A significant (Wilcoxon rank sum test, $p < 0.05$) increase was observed in the proportion of CD4-positive and CD8-positive cells over the 5 days of incubation. A significant decrease was observed for CD14-positive cells. There was no difference in the relative distribution of γδ T cells and B cells.

4.6. Discussion

The interaction between host and infectious agent determines the eventual outcome of the infection. In this study we investigated the influence of host infection status (test-positive versus test-negative for Johne's disease) and strain differences (5 strains, including the positive control) on the outcome of in vitro infection in MDMs.

Despite a century of research, Johne's disease remains one of the most mysterious and challenging infectious diseases of cattle. Intriguingly, only a small percentage of animals exposed to *M. paratuberculosis* can later be detected as infected with this pathogen (Johnson-Ifearulundu and Kaneene, 1999). This could be due to a difference in the ingested dose of the pathogen (Rankin, 1959) and/or the age of the animal at the time of exposure (Taylor, 1953). However, there is strong evidence that host factors are exceptionally important to susceptibility to mycobacteria (Bellamy, 2003). Several genes have been identified as potential determinants in *M. tuberculosis* infection. These include human natural resistance-associated macrophage protein gene (NRAMP1, recently renamed Slc11a1), and susceptibility to tuberculosis 1 locus (sst1). Mouse studies indicate there may be several additional genes associated with resistance to mycobacteria (tbs1 and tbs2, tuberculosis severity loci; Trl-1, Trl-2, Trl-3, Trl-4, tuberculosis resistance loci) (Bellamy, 2006). In *in vitro* experiments with multiple *M. tuberculosis* strains, Li et al. (2002) found substantial host-to-host variability in bacterial propagation.

As with *M. tuberculosis*, patterns of resistance to *M. paratuberculosis* may also be host-genetic related (Qureshi et al., 1996). There is evidence for inheritance of susceptibility to *M. paratuberculosis* infection in animals raised under the same conditions (Gonda et al., 2006; Koets et al., 2000; Mortensen et al., 2004; Nielsen et al., 2002). Furthermore,

Reddacliff et al. (2005) found an association between host MHC-II and NRAMP alleles with *M. paratuberculosis* clinical status in sheep.

In this study we compared intracellular survival of different *M. paratuberculosis* strains isolated from cattle and sheep in MDMs from peripheral blood of cows. We chose two groups of subjects to isolate MDMs from: animals that had repeatedly tested positive for Johne's disease and matched cows with negative results in all tests prior to the onset of experiments. To investigate potential variations in genetically determined susceptibility between cows of different infection status, we chose animals raised under the same management conditions from the same farm. As these animals had grown up in the same environment, we assume them to have been exposed to a similar microbial population. If this was true, then animals which were test-negative had either eliminated the agent or suppressed shedding to a level below the threshold of detection and thus are classified as resistant. Animals which shed *M. paratuberculosis* are less capable of fighting off infection and are termed susceptible.

We assumed that all MDMs used in this study were equally capable of phagocytosing bacteria. We accounted for the differences due to the experimental run by always pairing a test-positive with a test-negative animal and by using a linear mixed model for statistical analysis. Due to the experimental set-up we cannot estimate the proportion of live and dead bacteria using our counting data. However, comparison of real time PCR data with the microscopy data will allow addressing the question if there might have been a higher proportion of dead bacteria in the MDMs of test-negative animals compared to the cells of test-positive animals because in the PCR assay bacterial DNA of live and dead cells will be accounted for. Thus, potential differences might be demonstrated when interpreting the PCR data in the light of the findings of the currently reported experiments. In the described assay

we only kept track of live (green fluorescing) bacteria by utilizing carboxyfluorescein diacetate as an indicator of bacterial metabolic activity. The diacetate groups of the CFDA molecules are hydrolysed by intracellular esterases which results in the formation of the fluorescein molecules carboxyfluorescein; these are retained in cells. There is no esterase activity in non-viable cells, thus these cells do not fluoresce (Hoefel et al., 2003). Bacteria used for the assays were always prepared in the same manner. Thus, we assumed a high viability of bacteria and an equal proportion of live bacteria in the different infection media.

There was no effect of the origin of MDMs (resistant or susceptible animals) on phagocytosis, survival of bacteria, or macrophage survival in this dataset. This is in agreement with the findings of Weiss et al. (2005), who investigated the survival of one *M. paratuberculosis* strain in peripheral blood monocytes from infected and control animals from the same farm. However, this contrasts with findings of Khalifeh and Stabel (2004) which indicated that macrophages from infected cows are less capable of killing *M. paratuberculosis* than those from negative control animals.

The Weiss et al. study used a pure monocyte culture with no contact with autologous lymphocytes, while Khalifeh and Stabel used an unfractionated culture system with both macrophages and lymphocytes. Because our culture system closely resembled that of Khalifeh and Stabel, with a mixed culture at time of infection, we hypothesized that macrophages from subclinically infected animals would be less successful in killing *M. paratuberculosis* bacteria compared to cells from control cows.

The difference in the findings could be due to the small sample size in our study (n = 3 animals/group), resulting in a low power of statistical tests, or to the fact that we used only a population of subclinically infected animals, while Khalifeh and Stabel used both subclinical and clinical animals. In addition, we cannot rule out the possibility that our test-negative

animals had previously been exposed to or were infected with farm-specific *M. paratuberculosis* strains. It has been reported that between 30% and 47% of cattle that were consistently fecal culture-negative but originating from infected herds will have culture-positive tissues at the time of slaughter (Meyer zu Vilsendorf, 1995; Whitlock and Buergelt, 1996).

In the context discussed here, it may be that previous exposure to *M. paratuberculosis* is more important than current shedding status. Weiss et al. (2005) report that their control animals originated from the same farm as Johne's disease-positive animals, but that those cows did not show any signs of infection with *M. paratuberculosis* at necropsy (indicating a population that would have been classified as resistant in our study). Because Khalifeh and Stabel (2004) used animals from premises without any history of *M. paratuberculosis* as their control animals, those animals were not necessarily more resistant to infection, but had not been exposed. All of the positive animals in that study would have been classified as susceptible by our definition. A difference in cellular and humoral immunity of exposed and non-exposed animals was previously reported (Huda et al., 2004), but has not been evaluated in terms of killing capacity of macrophages.

M. paratuberculosis strains exhibit *in vivo* virulence differences at a host species level (Motiwala et al., 2006; O'Brien et al., 2006; Saxegaard, 1990). Although there are no specific proteins identified which explain species specificity, recent sequencing of the *M. paratuberculosis* genome (Li et al., 2005) and microarray analysis showed genome level differences between sheep and cattle strains (Marsh et al., 2006). Shin et al. (2006) identified by insertion sequence mutagenesis of several genes responsible for lower survival and distribution of *M. paratuberculosis* mutants in a mouse model. There are three conserved regions present in *M. avium* subsp. *avium* containing 24 open reading frames which are

present in cattle strains of *M. paratuberculosis* but lacking in sheep strains, and this may play a role in host specificity (Marsh et al., 2006). Host cytokine profiles, matrix metalloproteinases, and tissue inhibitor of matrix metalloproteinases differed when MDMs from naïve young cattle were infected with *M. paratuberculosis* strains isolated from human, ovine, or bovine hosts (Janagama et al., 2006).

There are few data on virulence differences between bovine-specific *M. paratuberculosis* strains *in vivo* or *in vitro*. Jaganama et al. (2006) noted a trend towards association of some MLSSR strain types with low morbidity.

We were interested in survival characteristics of *M. paratuberculosis* in MDMs from animals which had previously been naturally exposed to *M. paratuberculosis*. MDMs were infected with two *M. paratuberculosis* strains from the host farm, a bovine-specific strain which was from a different farm as well as an ovine-specific strain, and a non-*M. paratuberculosis*-positive control (member of the M. avium-intracellulare complex). Three of the 5 strains had previously been analyzed in naïve control animals (Janagama et al., 2006). In that study, bovine strain 1018 survived better than ovine strain 7565. Our additional bovine strains had SSR fingerprints (7G/6GGT and 7G/5GGT) which were distinct from that of strain 1018 (7G/4GGT). The assumption that the 7G/4GGT profile is one of reduced pathogenicity was testable and we found that the bovine strains were more successful at survival than the ovine and the positive control strains. Farm-specific strains were very similar in behavior to each other and more successful than bovine strain 1018.

Such strain-specific survival differences are well characterized in other mycobacterial species, with *M. tuberculosis* strains classified by their virulence in cell culture or in experimental infections of mice. Li et al. (2002) found that there is not a strong correlation between virulence as measured by CFU counts of bacteria in PBMC pure culture experiments

and in whole mouse models. Since we utilized a mixed culture MDM system, we expect that our results are more reflective of the immune status of the animal than when purified MDM cultures alone are used. Strain differences may result in some strains showing high levels of virulence or high contagiousness. However, these two characteristics (virulence and contagiousness) are likely independent and not necessarily both present in one specific strain. Tuberculosis strain CDC1551 is known to be a highly contagious, but relatively avirulent strain of *M. tuberculosis*, resulting in high rate of contacts testing positive for exposure, but in few individuals developing clinical signs (Manca et al., 2001). This is the first study showing potential virulence differences among bovine-specific *M. paratuberculosis* strains. Increased understanding of strain-specific behavior will make it possible to design more effective control strategies and could help answer some of the still open questions in Johne's research. If there are multiple strains present on a farm (Harris et al., 2006), there is the potential for strain competition, with multiple strains competing for the same niche in the host system. Due to the nature of our visual assays, we likely do not observe definite bacterial counts. However, all comparisons between animals and strains were made using the same technique, so we feel comfortable that our conclusions are valid.

Cytotoxicity of mycobacterial strains has been linked to virulence. Highly virulent strains of *M. tuberculosis* are able to suppress apoptosis of host macrophages, allowing bacterial survival and propagation (Raja, 2004). In our experiment, the number of macrophages for all cultures (including the uninfected culture) increased over time, probably as a result of continued maturation of monocytes to become adherent macrophages over the course of the experiment. It is possible that antigenic stimulation also caused proliferation in differentiated macrophages (Luo et al., 2005). MDMs infected with bovine *M. paratuberculosis* strains (1018, 1180, 1099) had a decrease in proliferation of adherent cells

between measurement times 3 and 4 relative to both controls (negative and positive control strain 6043) and MDMs infected with the ovine strain (7565). This would suggest that bovine *M. paratuberculosis* strains reduce fitness or maturation of host macrophages.

Although our study suggests that there are no obvious differences in macrophage killing capacity in test-positive and test-negative cows, the data cannot be used to draw conclusions on the animals' respective macrophage killing capacity as calves or as naïve adults exposed to the pathogen. It is the latter that more likely determines eventual infection status. This question could be efficiently explored with long-term longitudinal studies. This study demonstrated differences in phagocytosis and intracellular survival of four different genotypes of *M. paratuberculosis* isolates utilized in PBMC infection assays. The bacterial isolates from the farm on which the study animals were housed were more successful in invasion and survival in MDMs than bacterial strains to which the animals had not been previously exposed. We found no important differences in *in vitro* macrophage killing abilities between cows currently shedding *M. paratuberculosis* and matched test-negative controls. However, important differences were observed between strains of *M. paratuberculosis*. Nevertheless, the presented results need to be interpreted with caution due to the small number of animals used in our experiments. Further studies in this area would be essential to understand differences in pathogenesis and transmission characteristics of *M. paratuberculosis* strains and their associated impact on infection control programs.

4.7. Acknowledgements

We acknowledge the excellent technical support of Suzanne Klaessig. We also wish to thank Kenneth W. Simpson, Nancy A. Lorr, and Wilhelm H. Elmore for provision of laboratory facilities and technical support. Further we would like to thank Judy McAlister for

helpful discussions and critical reading of the manuscript. We also gratefully acknowledge the owner of the animals included in this study for his collaboration. Financial support for this work was provided in part by the USDA Agricultural Research Service (Agreement No. 58-1265-3-156) for the Regional Dairy Quality Management Alliance. Funding was also provided by the Johne's Disease Integrated Program (USDA contract 45105). Johne's disease research in SS laboratory is supported by JDIP and USDA-NRI grants.

4.8. Tables and Figures

Table 1: Characteristics of animals

	Animal ID					
	1180[a]	1184[a]	1160[b]	1194[b]	1179[c]	1038[c]
Test positive	Yes	No	Yes	No	Yes	No
Date of birth	11/17/00	11/26/00	08/19/00	12/06/00	11/11/00	06/12/99
Days in milk	318	330	432	321	385	696
Days pregnant at sampling	216	238	0	0	0	0

Blood samples for the isolation PBMC were taken on different dates. One test-positive and one test-negative animal were matched and sampled per post-infection measurement [a] 06/15/05, [b] 06/30/05, [c] 07/14/05

Table 2: **ELISA and Fecal Culture (FC) test results**

Test	Date	Animal ID					
		1180	1184	1160	1194	1179	1038
FC	02/04	11,6,10,4[a]	Neg	Neg	Neg	Neg	Neg
ELISA		0.462*	0.053	0.057	0.050	0.063	0.068
ELISA	06/04	0.756*	0.067	0.056	0.054	0.053	0.068
FC	09/04	75-150[b]	Neg	38,22,32,25 [a]	Neg	0,2,0,1 [a]	Neg
ELISA		2.512*	0.072	0.146	0.099	0.270*	0.075
FC	01/05	75-100 [b]	- [c]	150-200 [b]	- [c]	1,1,0,1 [a]	- [c]
ELISA		1.334*	0.064	0.537*	0.064	0.160	0.067
FC	04/05	150-200 [b]	Neg	75-100	Neg	3,7,7,3 [a]	Neg
ELISA		1.135*	0.072	0.754*	0.099	0.120	0.075
FC	07/05	100-200 [b]	- [c]	150-250 [b]	- [c]	0,7,0,6 [a]	- [c]
ELISA		1.237*	0.066	1.160*	0.056	0.336*	0.096

The Paracheck ELISA (CSL/Biocor, Omaha, NE) was used in this study; samples were considered positive (marked with *) if the final OD was greater than kit control plus 0.1.

The fecal culture test was performed as described by Crossley et al., 2005. Four tubes of Herrold`s egg yolk medium (HEYM) were inoculated with aliquots of each sample. Individual values[a] represent colony forming units (CFU) per tube, while a range of values[b] describes the total of CFU for all four tubes. On some test dates the FC was not performed for test-negative animals[c].

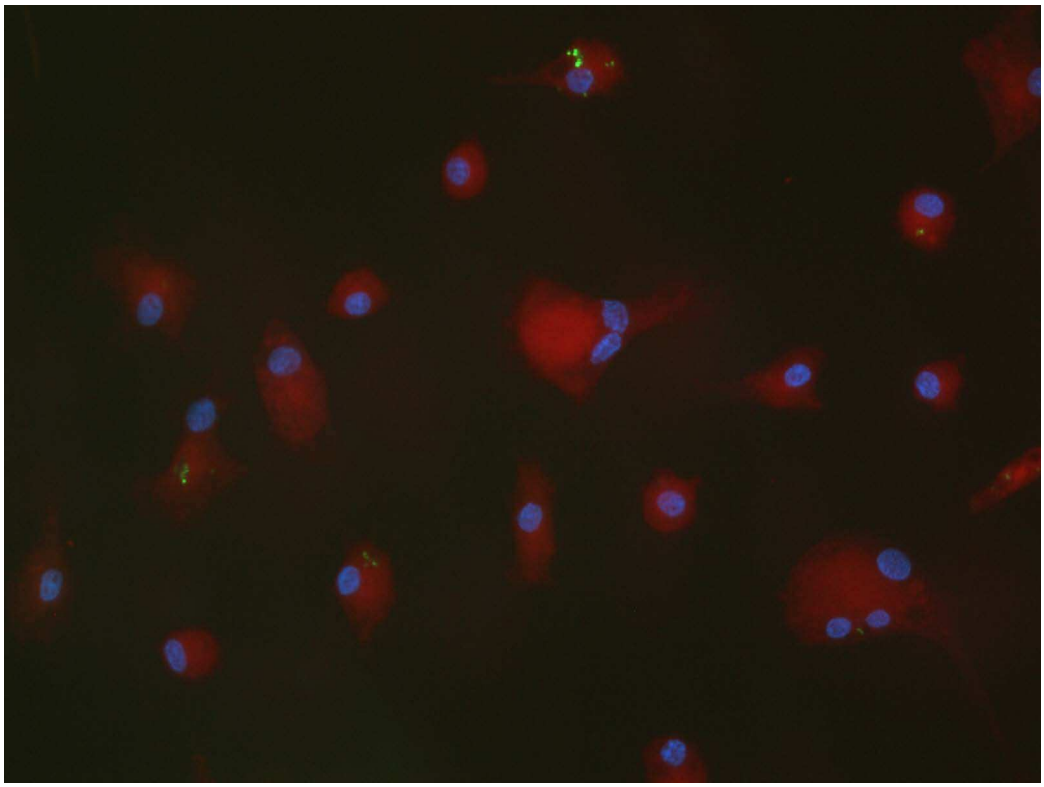

Fig. 1. Image of macrophages of cow 1194 infected with strain 1180 at post-infection measurement time 3. Visual fields were assessed at 400 x magnification with an Olympus System Microscope BX41 using the FITC, TRITC, and a DAPI filter. Three images were captured using different filters and merged in PictureFrame™ to be evaluated on the screen. MDMs (red) located completely within the boundaries of the picture were counted and assessed for bacteria (green). Infected macrophages were classified in five different categories according to the number of bacteria (zero, 1 to 10, 11 to 20, 21 to 50, and > 50 bacteria) per cell. For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.

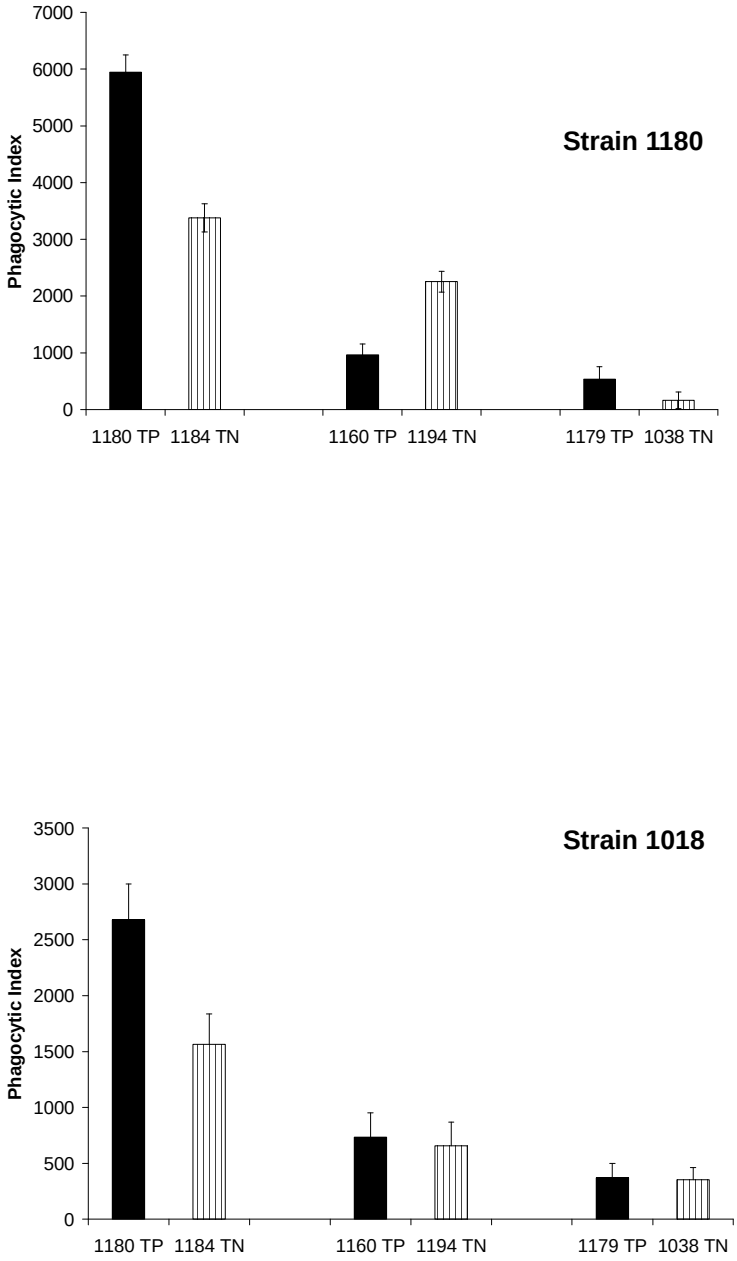

Fig. 2. Phagocytic index of MDMs infected with the indicated strains of *M. paratuberculosis* after 2 days of incubation. The test-positive (cow number followed by TP) and the test-negative (cow number followed by TN) animals are indicated along the horizontal axis. The animals are grouped in pairs reflecting the actual set-up of the experiment. No significant difference was observed between test-positive and test-negative cows.

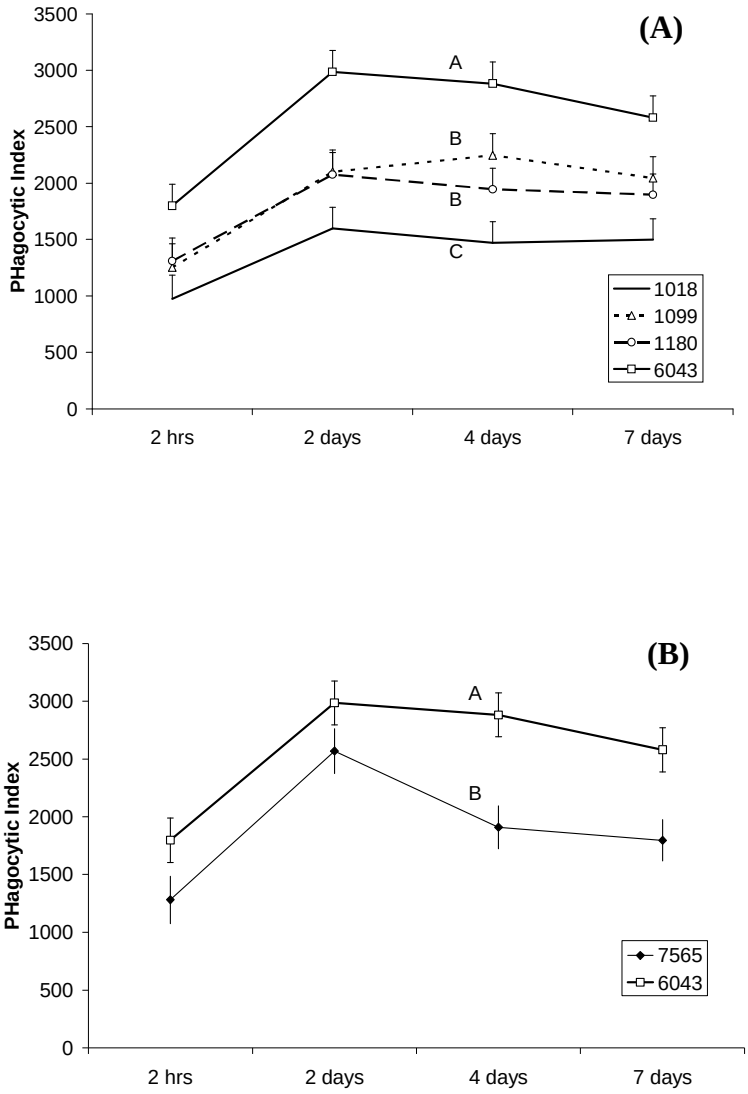

Fig. 3. Survival of the indicated strains of *M. paratuberculosis* and the positive control strain 6043 (member of the Mycobacterium avium-intracellulare complex) by MDMs over a period of 7 days. (A) Strain 1099 is the heterologous farm strain, strain 1180 is the homologous farm strain, and strain 1018 is an isolate from an Ohio dairy herd. Significant differences were observed between strains 6043, 1099, and 1180 and 1018, as indicated by different letters. (B) Strain 7565 is the ovine strain. Significant differences were observed between strains 6043 and 7565, as indicated by different letters. The results for (A) and (B) are presented as the phagocytic index (percent of infected cells times bacteria per infected cell).

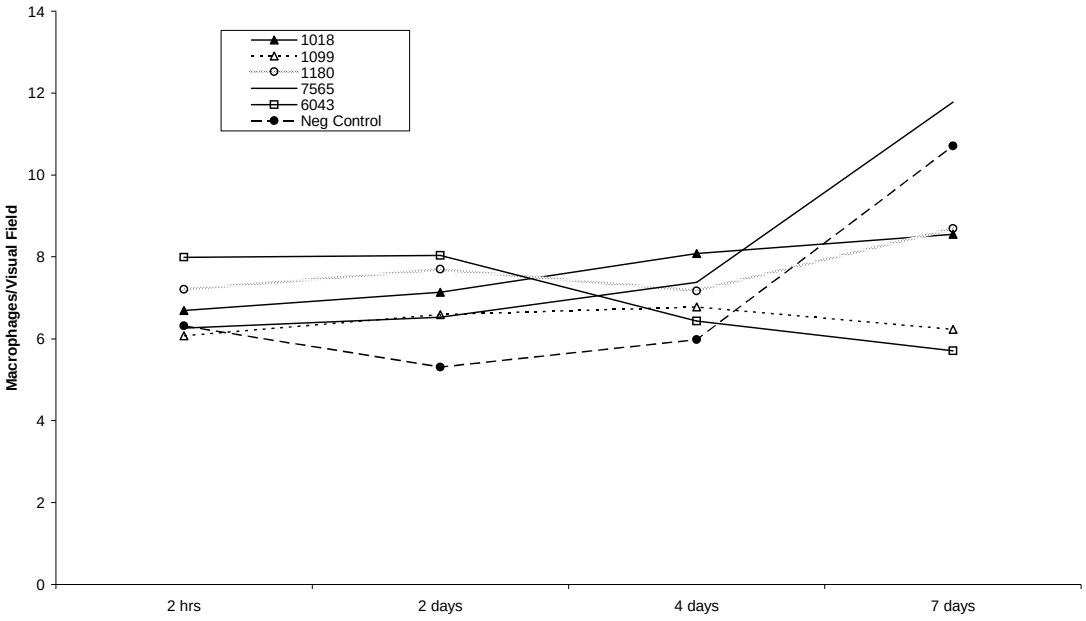

Fig. 4. Number of MDMs per visual field over a period of 7 days. All the bacterial strains and the negative control (medium only) are shown. MDMs infected with strains 6043 and 1099 had decreased proliferation of adherent cells between measurement times 3 and 4 relative to the other strains and the negative control.

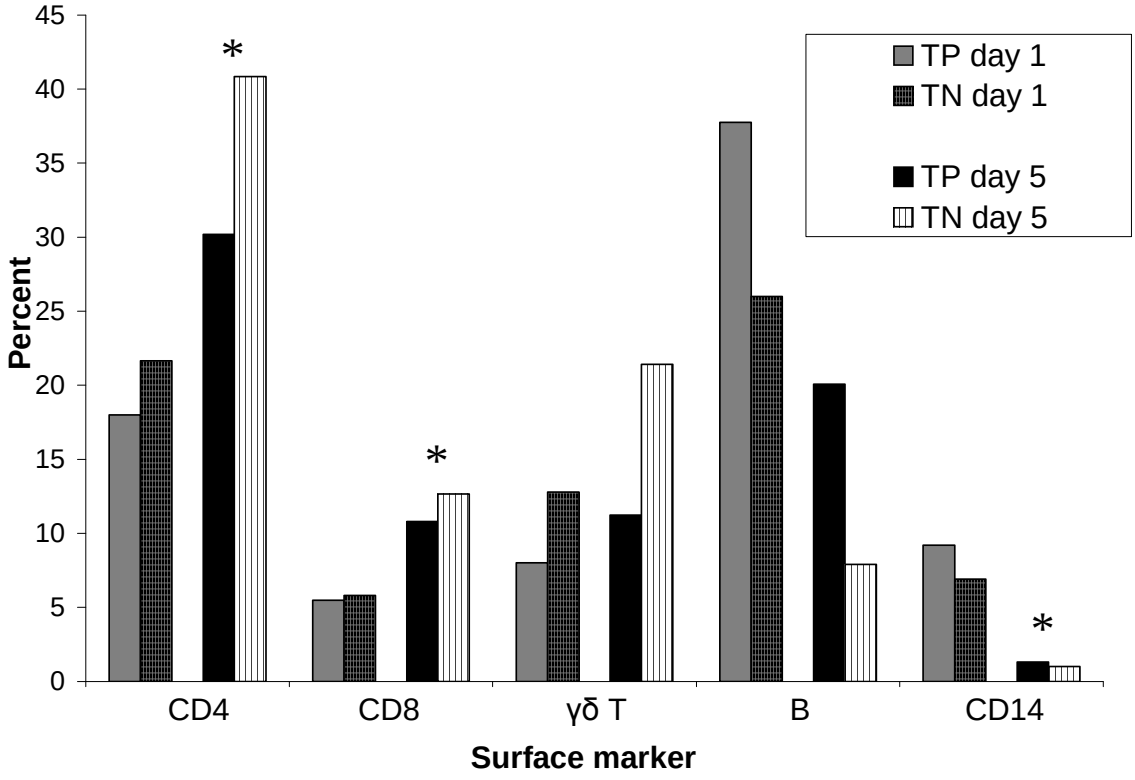

Fig. 5. FACS results of PBMC one day after isolation and after 5 days of incubation with autologous serum in Teflon containers just before experimental infection with mycobacteria. There was no significant difference between test-positive (TP) test-negative (TN) animals in any of the cell fractions on either day 1 or day 5. However, the relative distribution between day 1 and day 5 changed significantly ($p < 0.05$) for $CD4^+$, $CD8^+$ and $CD14^+$ cells.

4.9. References

Adams, L.G., Templeton, J.W., 1998, Genetic resistance to bacterial diseases of animals. Rev. Sci. Tech. 17, 200-219.

Akira, S., Uematsu, S., Takeuchi, O., 2006, Pathogen recognition and innate immunity. Cell 124, 783-801.

Amonsin, A., Li, L.L., Zhang, Q., Bannantine, J.P., Motiwala, A.S., Sreevatsan, S., Kapur, V., 2004, Multilocus short sequence repeat sequencing approach for differentiating among Mycobacterium avium subsp. paratuberculosis strains. J. Clin. Microbiol. 42, 1694-1702.

Badiola, J.J., Bakker, D., Garcia Marin, J.F., Gilot, P., Hermon-Taylor, J., Sharp, J.M., Shivananda, S., Thorel, M.F., Vuitton, D. 2000. Possible links between Crohn's disease and Paratuberculosis (European Commission, Directorate - General Health and Consumer Protection, SANCO/B3/R16/2000), p. 76.

Bang, B., 1906, Chronische pseudotuberkuloese Darmentzuendung beim Rinde. Berliner Tierärztliche Wochenschrift 42, 49-57.

Barksdale, L., Kim, K.S., 1977, Mycobacterium. Bacteriol. Rev. 41, 217-372.

Barthel, R., Feng, J., Piedrahita, J.A., McMurray, D.N., Templeton, J.W., Adams, L.G., 2001, Stable transfection of the bovine NRAMP1 gene into murine RAW264.7 cells: effect on Brucella abortus survival. Infect. Immun. 69, 3110-3119.

Barthel, R., Piedrahita, J.A., McMurray, D.N., Payeur, J., Baca, D., Suarez, G.F., Perumaalla, V.S., Ficht, T.A., Templeton, J.W., Adams, L.G., 2000, Pathologic findings and

association of Mycobacterium bovis infection with the bovine NRAMP1 gene in cattle from herds with naturally occurring tuberculosis. Am. J. Vet. Res. 61, 1140-1144.

Behr, M.A., Schurr, E., 2006, Mycobacteria in Crohn's disease: a persistent hypothesis. Inflamm. Bowel Dis. 12, 1000-1004.

Bellamy, R., 2003, Susceptibility to mycobacterial infections: the importance of host genetics. Genes Immun. 4, 4-11.

Bellamy, R., 2006, Genome-wide approaches to identifying genetic factors in host susceptibility to tuberculosis. Microbes Infect. 8, 1119-1123.

Bermudez, L.E., Young, L.S., Enkel, H., 1991, Interaction of Mycobacterium avium complex with human macrophages: roles of membrane receptors and serum proteins. Infect. Immun. 59, 1697-1702.

Blackwell, J.M., 2001, Genetics and genomics in infectious disease susceptibility. Trends Mol. Med. 7, 521-526.

Blackwell, J.M., Goswami, T., Evans, C.A., Sibthorpe, D., Papo, N., White, J.K., Searle, S., Miller, E.N., Peacock, C.S., Mohammed, H., Ibrahim, M., 2001, SLC11A1 (formerly NRAMP1) and disease resistance. Cell Microbiol. 3, 773-784.

Buergelt, C.D., Hall, C., McEntee, K., Duncan, J.R., 1978, Pathological evaluation of paratuberculosis in naturally infected cattle. Vet. Pathol. 15, 196-207.

Campbell, G.A., Adams, L.G., 1992, The long-term culture of bovine monocyte-derived macrophages and their use in the study of intracellular proliferation of Brucella abortus. Vet. Immunol. Immunopathol. 34, 291-305.

Chamberlin, W.M., Naser, S.A., 2006, Integrating theories of the etiology of Crohn's disease. On the etiology of Crohn's disease: questioning the hypotheses. Med. Sci. Monit. 12, RA27-33.

Chiodini, R.J., 1993, The History of Paratuberculosis (Johne's disease). A Review of the Literature 1895-1992. International Association for Paratuberculosis, 658 p.

Chiodini, R.J., 2005. The History of Paratuberculosis. In: Proceedings of Eighth International Colloquium on Paratuberculosis, Copenhagen, Denmark, pp. 1-4.

Chiodini, R.J., Van Kruiningen, H.J., Merkal, R.S., 1984, Ruminant paratuberculosis (Johne's disease): the current status and future prospects. Cornell Vet. 74, 218-262.

Clarke, C.J., 1997, The pathology and pathogenesis of paratuberculosis in ruminants and other species. J. Comp. Pathol. 116, 217-261.

Collins, M.T., 1996, Diagnosis of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 12, 357-371.

Coussens, P.M., 2001, Mycobacterium paratuberculosis and the bovine immune system. Anim. Health Res. Rev. 2, 141-161.

Coussens, P.M., Colvin, C.J., Rosa, G.J., Perez, L.J., Elftman, M.D., 2003, Evidence for a novel gene expression program in peripheral blood mononuclear cells from Mycobacterium avium subsp. paratuberculosis-infected cattle. Infect. Immun. 71, 6487-6498.

Coussens, P.M., Jeffers, A., Colvin, C., 2004a, Rapid and transient activation of gene expression in peripheral blood mononuclear cells from Johne's disease positive cows exposed to Mycobacterium paratuberculosis in vitro. Microb. Pathog. 36, 93-108.

Coussens, P.M., Pudrith, C.B., Skovgaard, K., Ren, X., Suchyta, S.P., Stabel, J.R., Heegaard, P.M., 2005, Johne's disease in cattle is associated with enhanced expression of genes encoding IL-5, GATA-3, tissue inhibitors of matrix metalloproteinases 1 and 2, and factors promoting apoptosis in peripheral blood mononuclear cells. Vet. Immunol. Immunopathol. 105, 221-234.

Coussens, P.M., Verman, N., Coussens, M.A., Elftman, M.D., McNulty, A.M., 2004b, Cytokine gene expression in peripheral blood mononuclear cells and tissues of cattle infected with Mycobacterium avium subsp. paratuberculosis: evidence for an inherent proinflammatory gene expression pattern. Infect. Immun. 72, 1409-1422.

Crossley, B.M., Zagmutt-Vergara, F.J., Fyock, T.L., Whitlock, R.H., Gardner, I.A., 2005, Fecal shedding of Mycobacterium avium subsp. paratuberculosis by dairy cows. Vet. Microbiol. 107, 257-263.

Dalziel, T.K., 1913, Chronic Interstitial Enteritis. British Medical Journal, 1058-1070.

Danelishvili, L., Wu, M., Stang, B., Harriff, M., Cirillo, S., Cirillo, J., Bildfell, R., Arbogast, B., Bermudez, L.E., 2007, Identification of Mycobacterium avium pathogenicity island important for macrophage and amoeba infection. Proc. Natl. Acad. Sci. U.S.A. 104, 11038-11043.

Daniels, M.J., Hutchings, M.R., Beard, P.M., Henderson, D., Greig, A., Stevenson, K., Sharp, J.M., 2003, Do non-ruminant wildlife pose a risk of paratuberculosis to domestic livestock and vice versa in Scotland? J. Wildl. Dis. 39, 10-15.

De Rosa, S. 2004. Protocol: Ethidium monoazide (EMA) for exclusion of dead cells by FACS, UW/Fred Hutchinson Cancer Reasearch Center, sderosa@fhcrc.org, http://www.microbiology.emory.edu/altman/f_protocols/protocolPDFs/EMA_procedure_DeRosa.pdf (last access: 09/12/06).

Dohmann, K., Strommenger, B., Stevenson, K., de Juan, L., Stratmann, J., Kapur, V., Bull, T.J., Gerlach, G.F., 2003, Characterization of genetic differences between Mycobacterium avium subsp. paratuberculosis type I and type II isolates. J. Clin. Microbiol. 41, 5215-5223.

Eichner Techau, M., Valdez-Taubas, J., Popoff, J.F., Francis, R., Seaman, M., Blackwell, J.M., 2007, Evolution of differences in transport function in SLC11A family members. J. Biol. Chem. [epub ahead of print] published on October 10, 2007 as manuscript M707057200.

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

Ferwerda, G., Kullberg, B.J., de Jong, D.J., Girardin, S.E., Langenberg, D.M., van Crevel, R., Ottenhoff, T.H., Van der Meer, J.W., Netea, M.G., 2007, Mycobacterium paratuberculosis is recognized by Toll-like receptors and NOD2. J. Leukoc. Biol. 82, 1011-1018.

Fridriksdottir, V., Gunnarsson, E., Sigurdarson, S., Gudmundsdottir, K.B., 2000, Paratuberculosis in Iceland: epidemiology and control measures, past and present. Vet. Microbiol. 77, 263-267.

Gilmour, N.J., Angus, K.W., 1976, The specificity and sensitivity of the fluorescent antibody test in cattle experimentally infected with Mycobacterium avium and Mycobacterium johnei. Res. Vet. Sci. 20, 6-9.

Gonda, M.G., Chang, Y.M., Shook, G.E., Collins, M.T., Kirkpatrick, B.W., 2006, Genetic variation of Mycobacterium avium ssp. paratuberculosis infection in US Holsteins. J. Dairy Sci. 89, 1804-1812.

Greger, M. 2001. Paratuberculosis and Crohn's Disease: Got Milk? http://www.mad-cow.org/00/paraTB.html (last access: 09/01/07).

Harris, N.B., Barletta, R.G., 2001, Mycobacterium avium subsp. paratuberculosis in Veterinary Medicine. Clin. Microbiol. Rev. 14, 489-512.

Harris, N.B., Payeur, J.B., Kapur, V., Sreevatsan, S., 2006, Short-sequence-repeat analysis of Mycobacterium avium subsp. paratuberculosis and Mycobacterium avium subsp. avium isolates collected from animals throughout the United States reveals both stability of loci and extensive diversity. J. Clin. Microbiol. 44, 2970-2973.

Hoefel, D., Grooby, W.L., Monis, P.T., Andrews, S., Saint, C.P., 2003, A comparative study of carboxyfluorescein diacetate and carboxyfluorescein diacetate succinimidyl ester as indicators of bacterial activity. J Microbiol Methods 52, 379-388.

Homuth, M., 2002, Diagnostik der Paratuberkulose. Dtsch. Tierärztl. Wochenschr. 109, 516-519.

Huda, A., Jungersen, G., Lind, P., 2004, Longitudinal study of interferon-gamma, serum antibody and milk antibody responses in cattle infected with Mycobacterium avium subsp. paratuberculosis. Vet. Microbiol. 104, 43-53.

Janagama, H.K., Jeong, K.I., Kapur, V., Coussens, P., Sreevatsan, S., 2006, Cytokine responses of bovine macrophages to diverse clinical Mycobacterium avium subspecies paratuberculosis strains. BMC Microbiol. 6, 10.

Jo, E.K., Yang, C.S., Choi, C.H., Harding, C.V., 2007, Intracellular signalling cascades regulating innate immune responses to Mycobacteria: branching out from Toll-like receptors. Cell Microbiol. 9, 1087-1098.

Johne, A., Frothingham, L., 1985, Ein eigenthuemlicher Fall von Tuberculose beim Rind. Dtsch. Z. Thiermed. Vergl. Path. 21, 438-454.

Johnson-Ifearulundu, Y., Kaneene, J.B., 1999, Distribution and environmental risk factors for paratuberculosis in dairy cattle herds in Michigan. Am. J. Vet. Res. 60, 589-596.

Johnson-Ifearulundu, Y., Kaneene, J.B., Lloyd, J.W., 1999, Herd-level economic analysis of the impact of paratuberculosis on dairy herds. J. Am. Vet. Med. Assoc. 214, 822-825.

Kennedy, D.J., Allworth, M.B., 2000, Progress in national control and assurance programs for bovine Johne's disease in Australia. Vet. Microbiol. 77, 443-451.

Kennedy, D.J., Benedictus, G., 2001, Control of Mycobacterium avium subsp. paratuberculosis infection in agricultural species. Rev. Sci. Tech. 20, 151-179.

Khalifeh, M.S., Stabel, J.R., 2004, Effects of gamma interferon, interleukin-10, and transforming growth factor beta on the survival of Mycobacterium avium subsp. paratuberculosis in monocyte-derived macrophages from naturally infected cattle. Infect. Immun. 72, 1974-1982.

Klee, W., Baumgartner, W., Stürner, M., 2002, Forensische Aspekte bei der Paratuberkulose des Rindes. Deutsches Tierärzteblatt 10, 1048-1049.

Koets, A., Rutten, V.P.M.G., Hoek, A., Bakker, D., van, Z.F., Muller, K., van, E.W., 1999. The T(h)1 to T(h)2 Shift in Bovine Paratuberculosis and the Role of Apoptotic Cell Death. In: Proceedings of Sixth International Colloquium on Paratuberculosis, Melbourne, Australia, 1999, pp. 585-592.

Koets, A.P., Adugna, G., Janss, L.L., van Weering, H.J., Kalis, C.H., Wentink, G.H., Rutten, V.P., Schukken, Y.H., 2000, Genetic variation of susceptibility to Mycobacterium avium subsp. paratuberculosis infection in dairy cattle. J. Dairy Sci. 83, 2702-2708.

Lambrecht, R.S., Carriere, J.F., Collins, M.T., 1988, A model for analyzing growth kinetics of a slowly growing Mycobacterium sp. Appl. Environ. Microbiol. 54, 910-916.

Levin, M., Newport, M., 2000, Inherited predisposition to mycobacterial infection: historical considerations. Microbes Infect. 2, 1549-1552.

Li, L., Bannantine, J.P., Zhang, Q., Amonsin, A., May, B.J., Alt, D., Banerji, N., Kanjilal, S., Kapur, V., 2005, The complete genome sequence of Mycobacterium avium subspecies paratuberculosis. Proc. Natl. Acad. Sci. U.S.A. 102, 12344-12349.

Li, Q., Whalen, C.C., Albert, J.M., Larkin, R., Zukowski, L., Cave, M.D., Silver, R.F., 2002, Differences in rate and variability of intracellular growth of a panel of Mycobacterium tuberculosis clinical isolates within a human monocyte model. Infect. Immun. 70, 6489-6493.

Luo, Y., Tucker, S.C., Casadevall, A., 2005, Fc- and complement-receptor activation stimulates cell cycle progression of macrophage cells from G1 to S. J. Immunol. 174, 7226-7233.

Manca, C., Tsenova, L., Bergtold, A., Freeman, S., Tovey, M., Musser, J.M., Barry, C.E., 3rd, Freedman, V.H., Kaplan, G., 2001, Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta. Proc. Natl. Acad. Sci. U.S.A. 98, 5752-5757.

Manning, E.J., Collins, M.T., 2001, Mycobacterium avium subsp. paratuberculosis: pathogen, pathogenesis and diagnosis. Rev. Sci. Tech. 20, 133-150.

Marsh, I.B., Bannantine, J.P., Paustian, M.L., Tizard, M.L., Kapur, V., Whittington, R.J., 2006, Genomic comparison of Mycobacterium avium subsp. paratuberculosis sheep and cattle strains by microarray hybridization. J. Bacteriol. 188, 2290-2293.

McKenna, S.L., Keefe, G.P., Tiwari, A., VanLeeuwen, J., Barkema, H.W., 2006, Johne's disease in Canada part II: disease impacts, risk factors, and control programs for dairy producers. Can. Vet. J. 47, 1089-1099.

Meyer zu Vilsendorf, U., 1995. Untersuchung zur Bekämpfung der Paratuberkulose mittels Merzung serologisch oder kulturell positiver Rinder. Dissertation. School of Veterinary Medicine, Hanover, Germany.

Mijs, W., de Haas, P., Rossau, R., Van der Laan, T., Rigouts, L., Portaels, F., van Soolingen, D., 2002, Molecular evidence to support a proposal to reserve the designation

Mycobacterium avium subsp. avium for bird-type isolates and 'M. avium subsp. hominissuis' for the human/porcine type of M. avium. Int. J. Syst. Evol. Microbiol. 52, 1505-1518.

Momotani, E., Whipple, D.L., Thiermann, A.B., Cheville, N.F., 1988, Role of M cells and macrophages in the entrance of Mycobacterium paratuberculosis into domes of ileal Peyer's patches in calves. Vet. Pathol. 25, 131-137.

Mortensen, H., Nielsen, S.S., Berg, P., 2004, Genetic variation and heritability of the antibody response to Mycobacterium avium subspecies paratuberculosis in Danish Holstein cows. J. Dairy Sci. 87, 2108-2113.

Motiwala, A.S., Amonsin, A., Strother, M., Manning, E.J., Kapur, V., Sreevatsan, S., 2004, Molecular epidemiology of Mycobacterium avium subsp. paratuberculosis isolates recovered from wild animal species. J. Clin. Microbiol. 42, 1703-1712.

Motiwala, A.S., Li, L., Kapur, V., Sreevatsan, S., 2006, Current understanding of the genetic diversity of Mycobacterium avium subsp. paratuberculosis. Microbes Infect. 8, 1406-1418.

Mullis, K.B., 1990, The unusual origin of the polymerase chain reaction. Sci. Am. 262, 56-61, 64-55.

NAHMS 1996. Part I: Reference of 1996 Dairy Management Practices. USDA:APHIS:VS, CEAH, National Animal Health Monitoring System, Fort Collins, CO.

NAHMS 1997. Johne's Disease on US Dairy Operations. USDA:APHIS:VS, CEAH, National Animal Health Monitoring System. Fort Collins, CO.

NAHMS 2007. Part I: Reference of Dairy Cattle Health and Management Practices in the United States, 2007. USDA:APHIS:VS, CEAH, National Animal Health Monitoring System, Fort Collins, CO.

Nielsen, S.S., Grohn, Y.T., Quaas, R.L., Agger, J.F., 2002, Paratuberculosis in dairy cattle: variation of the antibody response in offspring attributable to the dam. J. Dairy Sci. 85, 406-412.

O'Brien, R., Mackintosh, C.G., Bakker, D., Kopecna, M., Pavlik, I., Griffin, J.F., 2006, Immunological and molecular characterization of susceptibility in relationship to bacterial strain differences in Mycobacterium avium subsp. paratuberculosis infection in the red deer (Cervus elaphus). Infect. Immun. 74, 3530-3537.

Ott, S.L., Wells, S.J., Wagner, B.A., 1999, Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. 40, 179-192.

Payne, J.M., Rankin, J.D., 1961, A Comparison of the Pathogenesis of Experimental Johne's Disease in Calves and Cows. Res. Vet. Sci. 2, 175-179.

Pemberton, D.H., 1979, Diagnosis of Johne's disease in cattle using mesenteric lymph node biopsy: accuracy in clinical suspects. Aust. Vet. J. 55, 217-219.

Qureshi, T., Templeton, J.W., Adams, L.G., 1996, Intracellular survival of Brucella abortus, Mycobacterium bovis BCG, Salmonella dublin, and Salmonella typhimurium in macrophages from cattle genetically resistant to Brucella abortus. Vet. Immunol. Immunopathol. 50, 55-65.

Raja, A., 2004, Immunology of tuberculosis. Indian J. Med. Res. 120, 213-232.

Rankin, J.D., 1959, The estimation of doses of Mycobacterium johnei suitable for the production of Johne's disease in cattle. J. Pathol. Bacteriol. 77, 638-642.

Reddacliff, L.A., Beh, K., McGregor, H., Whittington, R.J., 2005, A preliminary study of possible genetic influences on the susceptibility of sheep to Johne's disease. Aust. Vet. J. 83, 435-441.

Reynolds, J.D., Morris, B., 1983, The evolution and involution of Peyer's patches in fetal and postnatal sheep. Eur. J. Immunol. 13, 627-635.

Rideout, B.A., Brown, S.T., Davis, W.C., Gay, J.M., Gianella, R.A., Hines, M.E., Hueston, W.E., Hutchinson, L.J., 2003, Diagnosis and Control of Johne's Disease. Committee on Diagnosis and Control of Johne's Disease, National Research Council, 244 p.

Russell, D.G., Mwandumba, H.C., Rhoades, E.E., 2002, Mycobacterium and the coat of many lipids. J. Cell Biol. 158, 421-426.

Saxegaard, F., 1990, Experimental infection of calves with an apparently specific goat-pathogenic strain of Mycobacterium paratuberculosis. J. Comp. Pathol. 102, 149-156.

Shin, S.J., Wu, C.W., Steinberg, H., Talaat, A.M., 2006, Identification of novel virulence determinants in Mycobacterium paratuberculosis by screening a library of insertional mutants. Infect. Immun. 74, 3825-3833.

Shulaw, W.P., Larew-Naugle, A., Torrence, M.E., Isaacson, R.E., 2003, Paratuberculosis: A Food Safety Concern?, In: Torrence, M.E., Isaacson, R.E. (Eds.) Microbial Food Safety in Animal Agriculture. Iowa State Press, Ames, IO.

Sohal, J.S., Singh, S.V., Subodh, S., Sheoran, N., Narayanasamy, K., Singh, P.K., Singh, A.V., Maitra, A., 2007, Mycobacterium avium subspecies paratuberculosis diagnosis and geno-typing: Genomic insights. Microbiol. Res. 2006; (2007), [epub ahead of print] doi:10.1016/j.micres.2007.1003.1005.

Stabel, J.R., 2000a, Cytokine secretion by peripheral blood mononuclear cells from cows infected with Mycobacterium paratuberculosis. Am. J. Vet. Res. 61, 754-760.

Stabel, J.R., 2000b, Johne's disease and milk: do consumers need to worry? J. Dairy Sci. 83, 1659-1663.

Stabel, J.R., 2000c, Transitions in immune responses to Mycobacterium paratuberculosis. Vet. Microbiol. 77, 465-473.

Stratmann, J., Homuth, M., Gerlach, G.F., 2005, Überlegungen zur Kontrolle und Bekämpfung der Paratuberkulose in Milchviehbeständen. Dtsch. Tierärztl. Wochenschr. 112, 304-306.

Streeter, R.N., Hoffsis, G.F., Bech-Nielsen, S., Shulaw, W.P., Rings, D.M., 1995, Isolation of Mycobacterium paratuberculosis from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. 56, 1322-1324.

Sweeney, R.W., 1996, Transmission of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 12, 305-312.

Sweeney, R.W., Whitlock, R.H., McAdams, S., Fyock, T., 2006, Longitudinal study of ELISA seroreactivity to Mycobacterium avium subsp. paratuberculosis in infected cattle and culture-negative herd mates. J. Vet. Diagn. Invest. 18, 2-6.

Sweeney, R.W., Whitlock, R.H., Rosenberger, A.E., 1992, Mycobacterium paratuberculosis isolated from fetuses of infected cows not manifesting signs of the disease. Am. J. Vet. Res. 53, 477-480.

Taylor, A.W., 1951, Varieties of Mycobacterium johnei isolated from sheep. J. Pathol. Bacteriol. 63, 333-336.

Taylor, A.W., 1953, Experimental Johne's disease in cattle. J. Comp. Pathol. 63, 355-367.

Thorel, M.F., Krichevsky, M., Levy-Frebault, V.V., 1990, Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of Mycobacterium avium, and description of Mycobacterium avium subsp. avium subsp. nov., Mycobacterium avium subsp. paratuberculosis subsp. nov., and Mycobacterium avium subsp. silvaticum subsp. nov. Int. J. Syst. Bacteriol. 40, 254-260.

Tizard, I.R., 2004, Veterinary Immunology: An Introduction, 7th Edition Edition. Elsevier.

Valeeva, N.I., Lam, T.J., Hogeveen, H., 2007, Motivation of dairy farmers to improve mastitis management. J. Dairy Sci. 90, 4466-4477.

van Roermund, H.J.W., Weber, M.F., de Koeijer, A.A., Velthuis, A.G.J., de Jong, M.C.M., 2005. Development of a milk quality assurance program for paratuberculosis: from within- and between-herd dynamics to economic decision analysis. In: Proceedings of Eighth International Colloquium on Paratuberculosis, Copenhagen, Denmark, pp. 51-59.

Viske, D., Larsson, B., Engvall, A., Bölske, G., 1996. Paratuberculosis in Sweden. In: Proceedings of Fifth International Colloquium on Paratuberculosis, Madison, Wisconsin, USA.

Wayne, L.G., Kubica, G.P., 1986, Bergey's manual of systematic bacteriology, Vol 2, 1 Edition. Holt, J. G., Sneath, P. H. A., Mair, N. S., Sharpe, M. E., 1435-1457 pp.

Weiss, D.J., Evanson, O.A., Souza, C.D., 2005, Expression of interleukin-10 and suppressor of cytokine signaling-3 associated with susceptibility of cattle to infection with Mycobacterium avium subsp paratuberculosis. Am. J. Vet. Res. 66, 1114-1120.

Wells, S.J., Ott, S.L., Seitzinger, A.H., 1998, Key health issues for dairy cattle - new and old. J. Dairy Sci. 81, 3029-3035.

Whitlock, R.H., Buergelt, C., 1996, Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet. Clin. North Am. Food Anim. Pract. 12, 345-356.

Whitlock, R.H., Wells, S.J., Sweeney, R.W., Van Tiem, J., 2000, ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. Vet. Microbiol. 77, 387-398.

Wu, C.W., Livesey, M., Schmoller, S.K., Manning, E.J., Steinberg, H., Davis, W.C., Hamilton, M.J., Talaat, A.M., 2007a, Invasion and persistence of Mycobacterium avium subsp.

paratuberculosis during early stages of Johne's disease in calves. Infect. Immun. 75, 2110-2119.

Wu, C.W., Schmoller, S.K., Shin, S.J., Talaat, A.M., 2007b, Defining the Stressome of Mycobacterium avium subspecies paratuberculosis in Vitro and in Naturally Infected Cows. J. Bacteriol.

Yasuda, M., Jenne, C.N., Kennedy, L.J., Reynolds, J.D., 2006, The sheep and cattle Peyer's patch as a site of B-cell development. Vet. Res. 37, 401-415.

Zadoks, R.N., Schukken, Y.H., 2006, Use of molecular epidemiology in veterinary practice. Vet. Clin. North Am. Food Anim. Pract. 22, 229-261.

Zhao, B., Collins, M.T., Czuprynski, C.J., 1997, Effects of gamma interferon and nitric oxide on the interaction of Mycobacterium avium subsp. paratuberculosis with bovine monocytes. Infect. Immun. 65, 1761-1766.

Zurbrick, B.G., Czuprynski, C.J., 1987, Ingestion and intracellular growth of Mycobacterium paratuberculosis within bovine blood monocytes and monocyte-derived macrophages. Infect. Immun. 55, 1588-1593.

5. Discussion

This chapter focuses especially on the discussion of the study design and prospects for future work. Further aspects of this doctoral study were elaborately discussed in chapter 4 (publication).

Many cows exposed to *M. paratuberculosis* will never reach the clinical stage of Johne's disease. In the majority of cases this is related to the long incubation period in which animals leave the herd due to other reasons such as failure of reproductive performance, or poor udder or claw health (McKenna et al., 2006). Furthermore, in some animals, the cellular immune response has been shown to be able to control the infection, with the animals never developing clinical signs of disease but remaining subclinically infected for life (reviewed by Coussens, 2001). Other cows infected with *M. paratuberculosis* even manage to efficiently eliminate the pathogen (Gilmour and Angus, 1976). Host factors such as age (Taylor, 1953), genetic constitution (Koets et al., 2000; Mortensen et al., 2004) and overall health condition (Kennedy and Benedictus, 2001) seem to be exceptionally important concerning the susceptibility to infection with *M. paratuberculosis*. Also environmental influences like poor housing conditions, bad climate, and social stress could make animals more prone to develop clinical disease. On the other hand, the infectious agent affects the outcome of infection. Dose of infectious agent (Rankin, 1959) and, likely, the genotypic characteristics of the infecting bacterial strain influence the infection (Bellamy, 2003; Motiwala et al., 2006).

This study addresses host-related and pathogen-related characteristics by experimental infection of MDMs with different strains of *M. paratuberculosis*. The potential differences in phagocytic and killing ability of MDMs as well as differences in intracellular survival of

different *M. paratuberculosis* strains in MDMs were investigated. MDMs for this study derived from cows which were test-positive by fecal culture and ELISA for *M. paratuberculosis*-infection and from matched animals of the same herd that had consistently shown negative results in both test categories. Four different *M. paratuberculosis* strains of different host specificity (bovine, ovine) and a control strain of the *M. avium-intracellulare* complex were used in the experiments.

The results did not lead to rejection of the hypothesis that the infection history of cows does not affect macrophage killing ability, while the hypothesis that killing capacity of bovine macrophages is not dependent on the strain had to be dismissed.

Finding no differences of survival of bacteria in MDMs between the two groups of animals may be attributable to a number of reasons:

- Certainly the low number of animals per group might mask slight differences between the groups due to relatively low power of statistical tests. Unfortunately the farm available for the presented study had a very limited number of suitable animals which influenced the study design. Otherwise conditions on the farm and the excellent data on the cows were ideal for the experiments. For future work the number of animals per group should be increased, within the constraints posed by available lab personnel and funding.
- All animals could be assumed to have been exposed to *M. paratuberculosis* and potentially infected with the pathogen, even though the control animals had never tested positive. Work by Meyer zu Vilsendorf (1995) and Whitlock and Buergelt (1996) demonstrated positive results of culture of lymph node tissue upon slaughter in animals which had never shown positive results for Johne's disease. Choosing control animals from a herd that has been tested repeatedly with uniformly negative results

could have yielded a different outcome of experiments and should be considered for future infection assays. Nonetheless, the choice of control group was based on the assumption that potential differences in MDM-activity of animals from the same background and the same environment would best reflect differences attributable to host-related characteristics.

- Cows used in this study were of the same breed and possibly of similar genetic constitution. However, this reflects the situation on farms world-wide where animals of similar genetic background show different outcomes of *M. paratuberculosis*-infections. Thus similar animals were intentionally chosen in order to detect any differences in macrophage behavior attributable to either infection status of the animal or variations of yet unidentified genes.
- *In vitro* studies can only try to mimic *in vivo* scenarios. The many factors influencing the *in vivo* immune reaction cannot be completely reproduced under laboratory conditions. Thus, it cannot be ruled out that even though macrophages were kept in the vicinity of the host-animals blood lymphocytes there was a crucial discrepancy in the communication between immune cells *in vitro* compared to cells *in vivo*. Unfortunately, this is a situation most basic research projects need to deal with and therefore calls for careful interpretation of results. In this study besides finding no significant differences in survival of bacteria in MDMs derived from the two groups of cows, differences in the leukocyte cell fractions determined by FACS could also not be detected. Thus far, cytokine profiles are not available but may further elucidate the state of cell communication in the presented experiments.

Differences in survival of *M. paratuberculosis* strains used in this study may be due to the following reason:

Even before strain typing was technically possible, epidemiological studies suggested that natural transmission between cattle and sheep was not common leading to the conclusion that the two species harbor different host adapted *M. paratuberculosis* strains (Fridriksdottir et al., 2000; Kennedy and Allworth, 2000). In recent years, genotypic differences between sheep and cattle strains as well as strains of other species have been demonstrated (Harris et al., 2006; Marsh et al., 2006; Motiwala et al., 2004), and molecular strain typing now becomes a valuable tool in epidemiological investigations of Johne's disease (Motiwala et al., 2006; Sohal et al., 2007). Different strains might possess different virulence properties which may influence the outcome of infection *in vivo* and *in vitro* (Janagama et al., 2006; Wu et al., 2007b). In the present study distinct differences of survival between the infecting bacterial strains (genotyped by MLSSR) were demonstrated. It may be concluded that survival differences of strains are attributable to their genotypic characteristics.

In conclusion, it is noted that the chosen study design was not optimal in order to detect possible differences in survival of bacteria in MDMs deriving from test-positive and test-negative cows. With regard to determination of potential genetic differences of cattle possibly leading to an increase of susceptibility for Johne's disease further studies with larger groups of animals should be conducted.

Furthermore, it may be suggested that strain differences of *M. paratuberculosis* might not only have impact on survival of the pathogen *in vitro* but also in natural hosts. This may have important implications on future investigation of Johne's disease in regard to:

- Interspecies transmission, especially by enlightening the possible infection of humans with animal strains. Additionally, further information is needed in regard to transmission between animal species which share the same habitat. Consequently, such

epidemiological studies may have an important impact on future control and food safety programs.

- Determination of virulence factors. This may help to answer some important questions concerning host-pathogen interaction and could become of great importance for Johne's disease eradication efforts.
- Gaining more information on the behavior of *M. paratuberculosis* strains within the same environmental niche, such as a dairy farm. This information could also contribute to improvement of management strategies for Johne's disease affected farms.

5.1. References

Bellamy, R., 2003, Susceptibility to mycobacterial infections: the importance of host genetics. Genes Immun. 4, 4-11.

Coussens, P.M., 2001, Mycobacterium paratuberculosis and the bovine immune system. Anim. Health Res. Rev. 2, 141-161.

Fridriksdottir, V., Gunnarsson, E., Sigurdarson, S., Gudmundsdottir, K.B., 2000, Paratuberculosis in Iceland: epidemiology and control measures, past and present. Vet. Microbiol. 77, 263-267.

Gilmour, N.J., Angus, K.W., 1976, The specificity and sensitivity of the fluorescent antibody test in cattle experimentally infected with Mycobacterium avium and Mycobacterium johnei. Res. Vet. Sci. 20, 6-9.

Harris, N.B., Payeur, J.B., Kapur, V., Sreevatsan, S., 2006, Short-sequence-repeat analysis of Mycobacterium avium subsp. paratuberculosis and Mycobacterium avium subsp. avium

isolates collected from animals throughout the United States reveals both stability of loci and extensive diversity. J. Clin. Microbiol. 44, 2970-2973.

Janagama, H.K., Jeong, K.I., Kapur, V., Coussens, P., Sreevatsan, S., 2006, Cytokine responses of bovine macrophages to diverse clinical Mycobacterium avium subspecies paratuberculosis strains. BMC Microbiol. 6, 10.

Kennedy, D.J., Allworth, M.B., 2000, Progress in national control and assurance programs for bovine Johne's disease in Australia. Vet. Microbiol. 77, 443-451.

Kennedy, D.J., Benedictus, G., 2001, Control of Mycobacterium avium subsp. paratuberculosis infection in agricultural species. Rev. Sci. Tech. 20, 151-179.

Koets, A.P., Adugna, G., Janss, L.L., van Weering, H.J., Kalis, C.H., Wentink, G.H., Rutten, V.P., Schukken, Y.H., 2000, Genetic variation of susceptibility to Mycobacterium avium subsp. paratuberculosis infection in dairy cattle. J. Dairy Sci. 83, 2702-2708.

Marsh, I.B., Bannantine, J.P., Paustian, M.L., Tizard, M.L., Kapur, V., Whittington, R.J., 2006, Genomic comparison of Mycobacterium avium subsp. paratuberculosis sheep and cattle strains by microarray hybridization. J. Bacteriol. 188, 2290-2293.

McKenna, S.L., Keefe, G.P., Tiwari, A., VanLeeuwen, J., Barkema, H.W., 2006, Johne's disease in Canada part II: disease impacts, risk factors, and control programs for dairy producers. Can. Vet. J. 47, 1089-1099.

Meyer zu Vilsendorf, U., 1995. Untersuchung zur Bekämpfung der Paratuberkulose mittels Merzung serologisch oder kulturell positiver Rinder. Dissertation. School of Veterinary Medicine, Hanover, Germany.

Mortensen, H., Nielsen, S.S., Berg, P., 2004, Genetic variation and heritability of the antibody response to Mycobacterium avium subspecies paratuberculosis in Danish Holstein cows. J. Dairy Sci. 87, 2108-2113.

Motiwala, A.S., Amonsin, A., Strother, M., Manning, E.J., Kapur, V., Sreevatsan, S., 2004, Molecular epidemiology of Mycobacterium avium subsp. paratuberculosis isolates recovered from wild animal species. J. Clin. Microbiol. 42, 1703-1712.

Motiwala, A.S., Li, L., Kapur, V., Sreevatsan, S., 2006, Current understanding of the genetic diversity of Mycobacterium avium subsp. paratuberculosis. Microbes Infect. 8, 1406-1418.

Rankin, J.D., 1959, The estimation of doses of Mycobacterium johnei suitable for the production of Johne's disease in cattle. J. Pathol. Bacteriol. 77, 638-642.

Sohal, J.S., Singh, S.V., Subodh, S., Sheoran, N., Narayanasamy, K., Singh, P.K., Singh, A.V., Maitra, A., 2007, Mycobacterium avium subspecies paratuberculosis diagnosis and geno-typing: Genomic insights. Microbiol. Res. 2006; (2007), [epub ahead of print] doi:10.1016/j.micres.2007.1003.1005.

Taylor, A.W., 1953, Experimental Johne's disease in cattle. J. Comp. Pathol. 63, 355-367.

Whitlock, R.H., Buergelt, C., 1996, Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet. Clin. North Am. Food Anim. Pract. 12, 345-356.

Wu, C.W., Schmoller, S.K., Shin, S.J., Talaat, A.M., 2007, Defining the Stressome of Mycobacterium avium subspecies paratuberculosis in Vitro and in Naturally Infected Cows. J. Bacteriol.

6. Summary

Survival of different *Mycobacterium avium* subsp. *paratuberculosis* strains in bovine monocyte-derived macrophages

Nicole Severine Gollnick

In this study the ability of different *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) strains to survive in bovine monocyte-derived macrophages (MDMs) was investigated. The following hypotheses were tested:

1) Infection status of a cow does not affect the ability of its macrophages to kill *M. paratuberculosis*
2) Killing capacity of bovine macrophages is not dependent on the *M. paratuberculosis* strain

MDMs for conducted experiments were obtained from Johne's disease-positive (n = 3) and age and stage of lactation matched Johne's disease-negative (n = 3) multiparious cows. Animals were kept on the same dairy operation, thus non-infected cows had been exposed to *M. paratuberculosis* strains present on the farm premises.

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation. After a five-day differentiation period in cell culture using Teflon jars MDMs were obtained and subsequently challenged *in vitro* with four *M. paratuberculosis* strains of different host specificity (bovine, ovine). MDMs were harvested at 2 hours, 2 days, 4 days and 7 days following infection. For each time point ingestion and

intracellular survival of *M. paratuberculosis* strains were determined by fluorescence microscopy.

There was no effect of the origin of MDMs (Johne's disease-positive or control animals) on phagocytosis, survival of bacteria, or macrophage survival. In contrast, important strain differences were observed. These findings suggest that some *M. paratuberculosis* strains interfere more successfully than others with the ability of macrophages to kill intracellular pathogens which may make it important to include strain typing when designing control programs.

7. Zusammenfassung

Überleben von verschiedenen *Mycobacterium avium* subsp. *paratuberculosis*-Stämmen in bovinen Makrophagen

Nicole Severine Gollnick

In dieser Studie wurde die Überlebensfähigkeit verschiedener *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) Bakterienstämme in primärer boviner Makrophagenkultur untersucht. Folgende Nullhypothesen wurden formuliert:

1) Der Infektionsstatus eines Tieres beeinflusst nicht die Fähigkeit der von diesem Tier stammenden Makrophagen, *M. paratuberculosis* abzutöten
2) Die Fähigkeit von Makrophagen, *M. paratuberculosis* abzutöten, ist nicht vom *M. paratuberculosis*-Bakterienstamm abhängig

Für die durchgeführten *in vitro* Versuche wurden mononukleäre Zellen aus dem peripheren Blut (PBMC) von Paratuberkulose-infizierten Kühen (n=3) und nicht-infizierten Kontrolltieren (n=3) gewonnen. Alle Tiere stammten aus demselben Betrieb und waren unter gleichen Umwelt- und Managementbedingungen aufgewachsen. Paratuberkulose-infizierte und nicht-infizierte Kontrolltiere wurden nach Alter und Laktationsstadium ausgewählt; Alle Tiere dieser Studie hatten die Möglichkeit zum Kontakt mit den auf der Farm vorkommenden *M. paratuberculosis*-Bakterienstämmen.

Unter Anwendung von Dichtegradientenzentrifugation und anschließender fünftägiger Kultivierung in Teflongefäßen wurden aus der monozytären Fraktion der Leukozyten

Makrophagen gewonnen. Im Folgenden wurden die Makrophagen mit vier verschiedenen *M. paratuberculosis*-Bakterienstämmen unterschiedlicher Wirtsspezifität (Rind, Schaf) infiziert. Nach 2 Stunden, 2 Tagen, 4 Tagen und 7 Tagen wurden Proben der Kulturen entnommen und auf Phagozytoserate und intrazelluläres Überleben von *M. paratuberculosis* mit Hilfe der Fluoreszenzmikroskopie untersucht.

Herkunft der verwendeten Makrophagen (Paratuberkulose-positive vs. Paratuberkulose-negative Tiere) hatte keinen Einfluss auf die Phagozytoserate und das Überleben der Bakterien. Unterschiede im Überleben von Makrophagen konnten ebenfalls nicht zwischen den beiden Gruppen festgestellt werden. Jedoch ließen sich signifikante Unterschiede im Überleben der verschiedenen Bakterienstämme nachweisen. Diese Ergebnisse geben einen Hinweis darauf, dass einige *M. paratuberculosis*-Stämme die Makrophagenfunktion stärker beeinträchtigen. Dies lässt es sinnvoll erscheinen, zukünftig im Rahmen von Paratuberkulose-Kontrollprogrammen die Genotypisierung von *M. paratuberculosis* in Betracht zu ziehen.

8. Curriculum Vitae

Personal details

Name:	Nicole Severine Gollnick
Date and Place of Birth:	June 1, 1975 in Varel, Germany
Nationality:	German

Education

10/96 – 08/02	**Veterinary Medicine**, School of Veterinary Medicine Hanover, Germany
09/99 – 07/00	**Veterinary Medicine**, Ecole Nationale Vétérinaire de Lyon, France
10/95 – 09/96	Carl von Ossietzky University Oldenburg, Germany **Majors:** English, Chemistry; **Minor:** Political Science
06/94	**Abitur**, Lothar-Meyer-Gymnasium Varel, Germany

Work experience

04/05 – present	**Resident**, Clinic for Ruminants, Veterinary Faculty of the University of Munich, Germany
10/03 – 03/05	**Intern**, Clinic for Ruminants, Veterinary Faculty of the University of Munich, Germany
07/03 – 09/03	**Visiting Clinician**, Veterinary Clinic Siepelmeyer and Erichsen, Nordenham, Germany
10/02 – 06/03	**Academic administrative co-ordinator** for the evaluation of the School of Veterinary Medicine Hanover by the European Association of Establishments for Veterinary Education (EAEVE) preparatory to the accreditation within the European Union, President‘s Office of the School of Veterinary Medicine Hanover, Germany
10/98– 06/99	**Project Assistant**, Vétérinaires Sans Frontières Germany, Hanover, Germany
07/94 – 08/95	**Au-Pair**, Philadelphia, PA, USA

9. Acknowledgements

Scientifically and personally the accomplishment of this thesis has been a terrific experience. I have had the chance to work and meet with many wonderful people without whom this project could not have been completed.

First and foremost, I would like to thank my thesis advisor Wolfgang Klee for giving me the opportunity to conduct my doctoral work abroad and for his guidance and constant support from beginning to end of this project. Likewise I thank my supervisor Ynte Schukken for inviting me to become a member of the “Johne’s girls” at Cornell University and for his tremendous support, advice and encouragement throughout the past years.

Further I would like to gratefully acknowledge all the people involved in the realization of this thesis project. It has been a great pleasure working with you! A special thank-you goes to Becky Mitchell for being an amazing co-worker and teacher! I also want thank Suzanne Klaessig for her exceptional technical assistance and help with all my quests and questions throughout the experimental period. I gratefully acknowledge Srinand Sreevatsan, Harish Janagama and Xiao Zhu at University of Minnesota for initiating me to the secrets of macrophage culture and for the pleasant and fruitful collaboration thereafter. Many thanks also to Kenny Simpson and his staff for letting me conquer their lab facilities. I especially like to thank Martin Baumgart for teaching me a few secrets about FACS. I also greatly acknowledge Nancy Lorr and Wilhelm Elmore – who gave me the wonderful opportunity to make myself at home at the Cornell teaching lab. I wish to thank Tracy French and the Cornell clinical pathology lab, Thomas Olson, Gerald Willis and Jim Smith for technical support. Further I want to thank Nancy Neilsen, Ute Schwab, and Thornik Reimer for helpful discussion on my project. I also like to thank David Green for his interest in my

work and the great collaboration. Further I want to thank Judy McAlister for editing the publication and for always believing in me.

These acknowledgements would not be complete without mentioning those who made me feel at home in Ithaca. Linda and Jeff Tikofsky, Ruth Zadoks, Hettie Schukken, Quirine Ketterings, Lisa Giacomelli, Andrea Rudella, Corina Vlot, Hanno van Raalte, Peter Blenkiron and Laila Geraci. Thank you for your friendship! And for all the gluestick providers and chocolate lovers at Quality Milk Production Services: It was a terrific time at 22, Thornwood Drive!

Last but not least I sincerely wish to thank my parents for their support and belief in me.