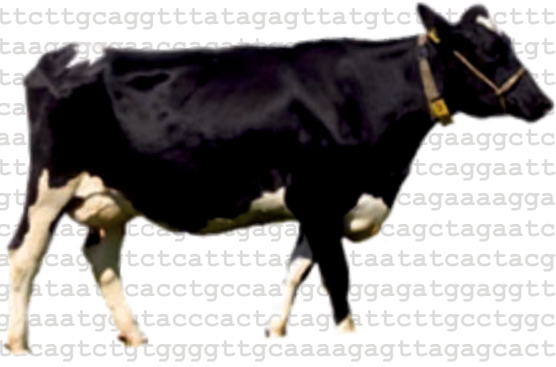
Genetic and phenotypic aspects of infection with *Mycobacterium avium* ssp. paratuberculosis in German Holstein cows

Julia Dorothee Küpper



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Die ganze Welt ist voll von Sachen, und es ist wirklich nötig, dass jemand sie findet.

(Astrid Lindgren)

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II ABBREVIATIONS

BGBI	Bundesgesetzblatt
BHV	*Bovine* herpes virus
BMVEL	Bundesministerium für Verbraucherschutz, Ernährung und Landwirtschaft
BTA	*Bos taurus*
BVD	*Bovine* Viral Diarrhea
CARD15	*Caspase Recruitment Domain*, *Member* 15
CD	Crohn‘s disease
cM	centimorgan
CXCL8	CXC-Motiv-Chemokin 8
CXCL10	C-X-C motif chemokine 10
CXCL1	Chemokine (C-X-C motif) ligand 1
DNA	Desoxyribonucleic acid
E. coli	*Escherichia coli*
e.g.	exempli gratia
ELISA	Enzyme-linked immunosorbent assay
et al.	et alia
GWA study	genome wide association study
HF	Holstein Friesian
IFN-γ	*interferon-gamma*

IL	*Interleukin*
JD	Johne's disease
Kbp	kilobase pair
MAP	*Mycobacterium avium* ssp. *paratuberculosis*
M. bovis	*Mycobacterium bovis*
Mbp	megabase pair
neg	negative
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NOD2	*Nucleotide Oligomerization Domain 2*
NRAMP1	natural resistance-associated macrophage protein 1
n.s.	not specified
PAMP	pathogen associated molecular pattern
ParaTB	Paratuberculosis
PCR	polymerase chain reaction
pos	positive
qPCR	quantitative polymerase chain reaction
QTL	Quantitative trait loci
Se	sensitivity
SLC11A1	*solute carrier family 11 member 1*
SNP	single nucleotide polymorphism
Sp	specificity
TLR	*Toll-like receptors*
TNF-α	Tumor Necrosis Factor-α
US	United States

VIT	Vereinigte Informationssysteme Tierhaltung w.V.

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1. General Introduction:

6. General Discussion and Conclusion

1. General Introduction

Paratuberculosis (ParaTB) or Johne's disease (JD) is caused by Mycobacterium avium subspecies paratuberculosis (MAP) and is reported in ruminants worldwide, particular from continents that have ruminants populations in husbandry. Relating to industry concern and economic impact Davis *et al*. (2009) ranked ParaTB on the same level as mastitis, *Bovine* Viral Diarrhea (BVD) and *E. coli* infections. Beside bovine, infections with MAP are reported especially in sheep and goats, but also in a range of deer species in the wild, in parks, in enclosures, and in zoos in different countries. So, in Europe, for example, ParaTB has been diagnosed in fallow, red and roe deer, reindeer, and moos (reviewed in Mackintosh and Griffin, 2010). Furthermore, ParaTB was reported in bison, riverine buffalo, bighorn sheep, mouflon, various antelope species, llamas, and alpacas (reviewed in Mackintosh and Griffin, 2010). ParaTB has been considered as infection of ruminants, but mainly in the last 15 years it has been confirmed, that non-ruminants can be a host of ParaTB. So far, classical clinical cases like in ruminants do not usually exhibit in non-ruminants. It is assumed that subclinical infected animals will be infected for the whole life and shed the agent intermittently in feces and spread the bacterial. Therefore, the wildlife must be included in the disease control strategies (Carta *et al*., 2013). An infection with MAP in non-ruminants could be demonstrated worldwide in different species, examples of different studies are summarized in Appendix Table 1.

Additionally, there is an ongoing controversial discussion about a possible link between Johnes's disease and Crohne's disease (CD) in humans, whereas the causal association is so far unknown. CD is a painful, chronic, inflammatory disease of the gastrointestinal tract, with chronical diarrhea, wasting, and mucosal appearance. In 1913, Dalziel observed the resemblance between the symptoms in bovine JD and human CD. Chiodini *et al*. reported in 1984 for the first time the isolation of MAP in a CD patient (reviewed by Chiodini, 1989). Since then, the verification of MAP succeeded in different types of tissue (e.g. Bull *et al*., 2003; Autschbach *et al*., 2005; Romero *et al*., 2005; Sechi *et al*., 2005), in blood (Naser *et al*., 2004), and in breast milk of two CD patients (Naser *et al*., 2000). However, in some of these studies the mycobacterium was also found in CD negative patients: for example, Bull *et al*. (2003) found MAP in 26% of the controls, Autschbach et al. (2005) found MAP in 2 – 5% of controls, and Sechi *et al.* (2005) found the organism in 10% of the controls. Other studies did

not find any evidence for the presence of MAP in CD patients (Wu et al., 1991; Rowbotham et al., 1995).

1.1 Paratuberculosis in bovine

ParaTB in bovine was first described by Johne and Frothingham in 1895 as a "peculiar case of tuberculosis". Bang (1906) recognized that the disease was not tuberculosis and renamed it pseudotuberculosis or Johne's disease. Soon afterwards, the causative bacillus for the meanwhile named was cultured, characterized and could also be used for experimental infections (Clark, 1997).

Agent

The causative agent MAP is an acid-fast, aerobic, weakly gram positive bacillus of 0.5 – 1.5 µm length and belongs to the Mycobacterium avium complex (Bisping and Amtsberg, 1988). The extremely slow growth and inability to produce mycobactin distinguish MAP from other Mycobacterium species. Other characteristic differences are the insertion of the element IS*900* that occurs with 14 – 18 copies within the MAP genome (Collins *et al*., 1989). The bacterial cell wall is complex, nearly impermeable, comprises glycolipids, peptidoglyolipids (mycosides) and long chain fatty acids (mycolic acids), which allowed the "Ziehl-Neelsen Färbung" for detection and gives MAP the acid-fast properties. Furthermore, MAP is resistant to dry, acid conditions, numerous disinfectants and can survive in water, feces and soil for months (Chiodini *et al*., 1984).

Infection

The probably most important mechanism for MAP transmission (Figure 1) is the fecal-oral route from the infectious adult cattle to susceptible calves (Sweeney, 1996). The long incubation period of the disease allows shedding of MAP before clinical symptoms appear, whereas the shedding of the agents is particularly high (up to $5x10^{12}$ mycobacteria per day)

from animals in the clinical phase of disease (Chiodini *et al*., 1984). Thereby, doses of 10^4 organisms per calve seems to be enough for infection (Gerlach, 2002), whereas with higher doses of the agent the risk of infection increases.

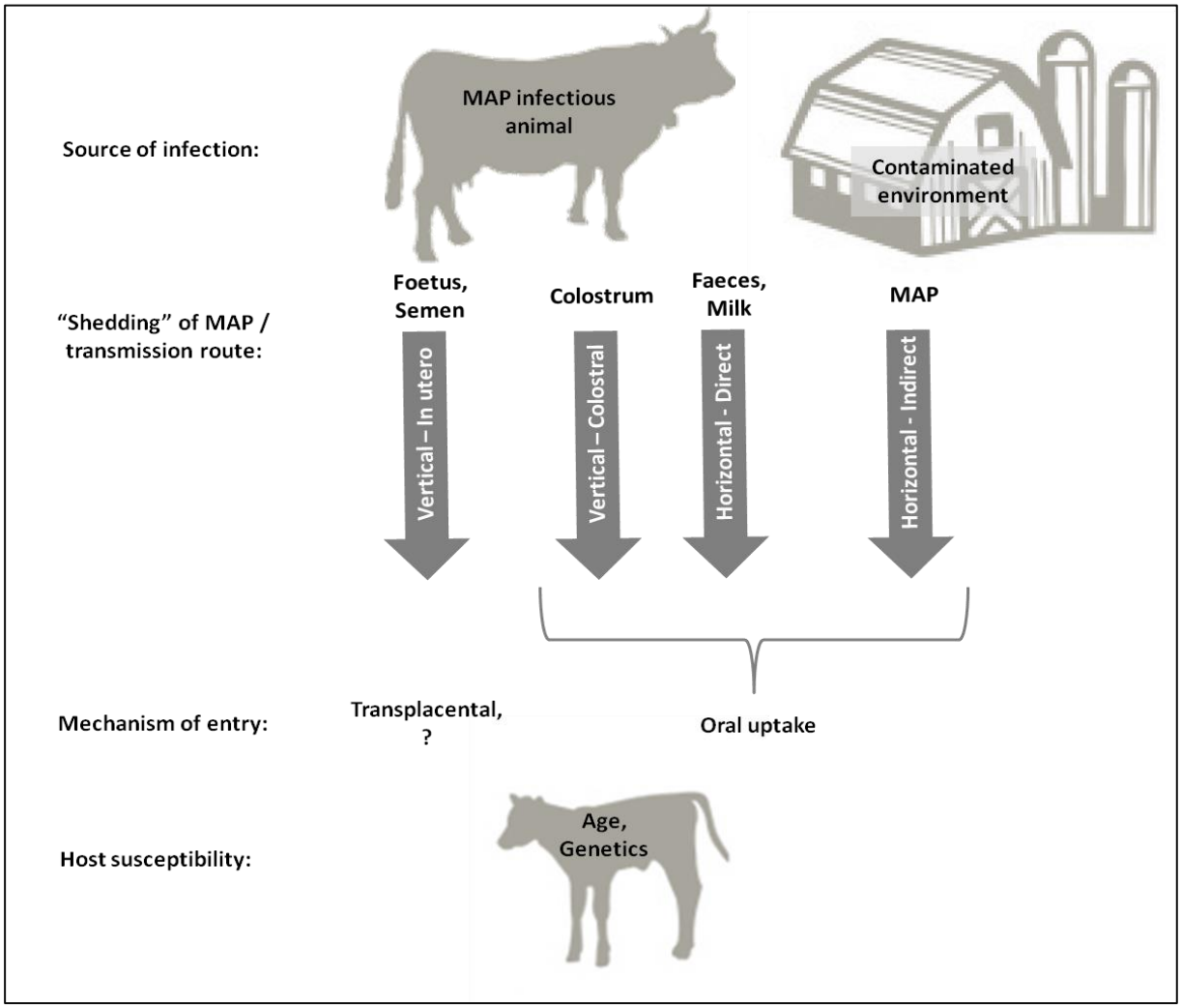

Figure 1: Routes and influence factors of MAP transmission.

Fecal contamination of teats, water, hay, and other feed resources as well as the contamination of the environment may cause to ingest large doses of the organism in calves (Sweeney, 1996). Thereby, the susceptibility of infection is highest in newborns and decreases with age (Windsor and Whittington, 2010).

Beside the transmission via adult cattle, van Roermund *et al*. (2007) reported that calves infected other calves. In addition, to the fecal-oral route, intra-uterine transmission and transfer via colostrum and milk have been reported (Chiodini *et al*., 1984; Sweeney *et al*.,

1992a, 1992b). However, the risk of the intrauterine infection seems to be low. Only 9% of fetuses from subclinical infected cows and 39% of fetuses from clinical infected cows sickened (Whittington and Windsor, 2009).

Clinical signs

The incubation time after MAP infection can be up to 15 years. The most cases occur between 3 – 5 years of age, till then affected animals show no clinical symptoms like chronic progressive weight loss with chronic or intermitted diarrhea (Chiodini *et al*., 1984). Important is the differentiation between infection and disease: a MAP infection does not always result in ParaTB. Parturition, lactation and other stress are triggering factors for clinical disease. Whitlock and Buergelt (1996) divide Johne's disease into four different stages:

First stage: "silent infection", a long latent stage, without clinical signs after infection with MAP. Humoral response as well as MAP excretion is not detectable (Cocito *et al*., 1994). Only the cellular immune response measured by interferon gamma production of mononuclear cells seems to be a useful tool for the identification of subclinical infected cows (Stabel, 1996). However, due to test characteristics and logistic constraints, this test is not commonly used under field conditions.

Second stage: after 2 – 5 years, the cows enter the subclinical stage. In this stage a humoral response starts to develop and affected animals shed the agent intermittently.

Third stage: a decrease in milk production, chronic diarrhea, and weight loss are typical clinical signs and characterize the third stage (clinical stage) of disease. Also, this stage comes along with heavy shedding of the bacterium and detectable humoral responses.

Fourth stage: most animals are culled before reaching clinical disease. This stage is characterized by increasing lethargy, emaciation and profuse diarrhea up to the point of death.

1.1.1 Diagnostic

Diagnostic of MAP infection is one of the main problems as different diagnostic tests have limitations due to different sensitivity and specificity. The sensitivity and specificity compared to a test that identifies animals as truly infected or truly non-infected ("Gold standard") is a degree for effectiveness (Gilardoni *et al*., 2012). The "Gold standard" in the case of ParaTB is the fecal culture method (Chacon *et al*., 2004), which is not either 100% accurate to identify truly infected animals, especially because of the lack of shedding in the early phases of infection followed by the intermitted shedding. The effectiveness of a diagnostic test is also given by the positive and negative predictive value, which is influenced by the prevalence, so the risk of misclassification of individual cows is higher in low prevalence herds (Hendrick *et al*., 2005a).

Rough, there are two different possibilities of ParaTB identification, the direct diagnosis of the agent or part of it, and the indirect diagnosis of the immune response to the agent (Nielsen *et al*., 2002a; Chacon *et al*., 2004). Possibilities for the direct diagnosis of the causative agent are the bacterial culture (from feces, colostrum, milk, mucosal scrapings, and tissue), the microscopy, and the identification of genetic material of the agent. Enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion, intradermal reaction, detection of interferon gamma, complement fixation, flow cytometry, and histopathological examination are indirect diagnostic methods (Table 1; reviewed by Gilardoni *et al*., 2012). Because of the reduction in time needed compared to fecal culture, the direct polymerase chain reaction (PCR) is becoming more popular as diagnostic method for paratuberculosis, whereas the sensitivity and specificity is comparable to the fecal culture test method (Douarre *et al.*, 2010; Timms *et al*., 2011). The challenging on PCR techniques are especially the DNA extraction from difficult matrix such as feces because of the presence of PCR inhibitors and thick waxy cell wall of MAP (Timms *et al*., 2011). So, the most frequent used tests are the bacterial culture of fecal samples and the ELISA test (Nielsen *et al*., 2002a).

The bacterial culture can be performed depending on the question, on individual animals, on pooled samples, or on samples from the environment (Donat *et al*., 2011). The benefit for the culture of the agent from feces is the specificity of almost 100% (Nielsen and Toft, 2008), whereas the sensitivity is wide ranged, depending on the classification of infection or clinical

stage: between 23 – 29% in infected animals, up to 70% in affected, and 74% in infectious animals (Nielsen and Toft, 2008), or between 45 – 72% in subclinical stage, and up to 91% in the clinical stage of infection (Gilardoni *et al*., 2012). This means that to be sure about identity of bacterial growth, it usually needs to be confirmed by molecular methods (Whittington *et al*., 1998). Another handicap of the bacterial culture from feces is the long cultivation time, 4 – 8 weeks for liquid systems and 8 – 16 weeks for solid media (Sweeney *et al*., 2012), and therefore the high costs.

Table 1: Direct and indirect test methods beside bacteriological culture (from feces, colostrum, milk, mucosal scrapings, and tissue) and ELISA (serum and milk) for infection with *Mycobacterium avium* ssp. *paratuberculosis* (Gilardoni *et al*., 2012).

	Sample	Method	Detecting of	Comments
Direct	Feces, Colostrum, Milk, Tissue	Bacterioscopic	Agent	+[1] = simple, fast, inexpensive -[2] = low Se and Sp
	Milk Feces, Blood, Tissue, Lymph nodes	PCR (e.g. IS*900*)		- = high costs, false pos. Results (contamination during the technique), false negative (possible inhibitory components on Taq polymerase)
Indirect	Plasma	ELISA IFN-γ	Cell-mediated or humoral immune response-	Sp = 98% subclinical stage: Se = 41% + = detection of subclinical stage possible: - = cross reaction, high costs, quick handling of samples,
	Serum	Complement fixation		Sp = 96.7% silent stage: Se = 95.2% - = difficult to implement
	Serum	Agarose gel immuno-diffusion		+ = fast, inexpensive, easy to implement - = low Se
		Intradermal reaction		Sp = 79% subclinical stage: Se = 54%
	Serum	Flow cytometry		Sp = 97% Se = 95% + = fast - = expensive, complex to excute, sophisticated equipment required
	Tissue Lymph nodes	Histopathological evaluation	Histopathological examination	+ = identifies animals in subclinical stage - = expensive, requires trained personnel

[1]= pro, [2]= contra
Sp = specificity, Se = sensitivity

Controversial discussed is the probability of false positive fecal culture test results because of “pass through” of MAP. The assumption is that in certain animals the bacterium cannot colonize the gastrointestinal tract. So the relevant animals shed the agent with feces days after intake (Sweeney *et al*., 1992c; Fecteau *et al*., 2010). This phenomenon is assumed in highly infected herds. Gilardoni *et al*. (2012) argue that it seems to be impractical that enough bacteria for a false positive fecal culture test can pass the whole digestive system complex of ruminants without degradation.

On the other side, the ELISA is a cost efficient and rapid diagnostic test with high-throughput potential through automation (Collins *et al*., 2005) and hence used for herd screening. The test can be applied with blood serum and milk, due to a moderate correlation between them (Hendrick *et al*., 2005b). The main disadvantage of the ELISA test is the low sensitivity and the cross-reactivity with mycobacteria other than MAP (Harris and Barletta, 2001). In general, the probability of being ELISA-positive is different across lactation and number of parity (Nielsen *et al*., 2002b). The described sensitivity of ELISA ranges between 7% in the silent stage of disease, 15% in the in the subclinical stage, and up to 85 – 98% in the clinical stage of infection (Whitlock *et al*., 2000; McKenna *et al*., 2005a, 2005b; Nielsen and Toft, 2008; Gilardoni *et al*., 2012), or respectively 75% in heavy shedders and 26% in moderate and light shedders (Sweeney *et al*., 2006). The diagnostic specificity is between 87.9% and 99.8% (Whitlock *et al*., 2000; McKenna *et al*., 2005a, 2005b). To increase the sensitivity, especially in low-prevalence herds, it is the recommendation to combine ELISA test and the detection of the bacterium by culture and PCR (Gasteiner *et al*., 2000).

1.1.2 Prevalence

In bovine the reported MAP-prevalence varies between countries and regions. Also because of the different test methods used for MAP infection, the comparison of the unique prevalence studies is difficult, as the prevalence is affected by the accuracy of the used diagnostic test (Nielsen and Toft, 2009). Especially for the detection of animals in the latent stage of the disease ELISA-tests are inadequate (Sweeney *et al*., 1995). Therefore, the usage of serological tests alone allows only the identification of a small proportion of infected

animals (Köhler *et al*., 2008). In contrast, the sensitivity of fecal culture depends on the stage of the disease. Especially in the early stage of infection the affected animals secrete the agent intermittently (Whitlock and Buergelt, 1996) and above all, infected animals shed the MAP organism to a different extent (Whitlock *et al*., 2000). Caused by shedding under the detection limit, this leads to false-negative test results.

In the USA a prevalence for dairy herds of 66% to 70% and a within-herd prevalence of 5.6 – 8.0% (Johnson-Ifearulundu and Kaneene, 1998; Lombard *et al*., 2013) as well as a seroprevalence of 3.0% for beef cattle and 43.8% for beef cattle herds (Roussel *et al*., 2005) are reported. In Uganda Okuni *et al*. (2011, 2013) reported a prevalence of 3.7% for ELISA positive animals and respectively 4.7% of the animals with ParaTB characteristic granulomas. 33.8% of 62 herds with at least one ELISA positive animal were described, whereas the prevalence of MAP antibodies was different among the breeds: 8.9% in Holstein Friesian, 3.7% in Zebu, 1.4% in Ankole, 9% in Guernsey, and 0% in Ayrshire (Okuni *et al.*, 2011). In Europe, Nielsen and Toft (2009) reviewed the prevalence of MAP infection at the herd level of approximately >50%. The prevalence among cattle ranged between 2% and 24%, depending on the study and country. Results of prevalences from randomly selected studies in Germany are sumarized in Table 2 and from randomly selected studies in different European countries are summarized in Appendix Table 2.

Table 2: Prevalences in Germany for infection with *Mycobacterium avium* ssp. *paratuberculosis* in cattle.

Region	Prevalence (%)	Level	Test	Age-group	N =	Reference
Saxony	Ø 9.6	animal	ELISA[1]	n.s.[+]	55 394	Donat *et al.* (2005)
	dairy: 8.4 beef: 1.8	animal	ELISA[1]		49 herds 8 herds	
	95.3	herd	ELISA[1]			
Thuringia	Ø 9.5 (1.7 – 58.8)	animal	fecal culture		13 952	Donat *et al*., (2012)
North Rhine-Westphalia	16.8 1.7	animal	ELISA[2] tissue and fecal culture	culled cattle > 18 months	536	Böttcher (1997)
Bavaria	1.5 0.9 24.0	animal	ELISA[1] ELISA[3] ELISA[4]	n.s.	2 748	Böttcher and Gangl (2004)
	93.0-98.0 20.0-45.0 12.0-18.0	herd	ELISA[1] ELISA[3] ELISA[4]			
Mecklenburg-West Pomerania	12.2	animal	ELISA[1]	> 20 months	2 997	Hacker *et al.* (2004)
	84.7	herd	ELISA[4]	> 20 months	2 997	

[1]IDEXX, [2]in-house, [3]Pourquier, [4]Svanovir,
[+]not specified

1.1.3 Economic losses

The economic losses for Johne's disease are due to a reduction in milk and fattening performances (McKenna *et al*., 2006), increased calving intervals, decreased fertility (Johnson-Ifearulundu *et al*., 2000), and higher feed intake (Raizman *et al*., 2009). Other factors like costs associated with the management of infected animals (McKenna *et al*., 2006) and permanent culling to decrease the risk of infection (Raizman *et al*., 2009) can also cause economic losses.

Different authors determined the height of economic losses by different approaches. Ott *et al*. (1999) compared dairy herds with and without different levels of MAP infection, and estimated annual losses of around 200 to 250 million US$ for the US dairy industry. Chi *et al*. (2002) estimated 2 472 US$ annual losses for an average, infected, 50 cow herd and Raizman *et al*. (2009) estimated losses of 276 US$/per cow for fecal positive cows vs. negative ones

because of deficit of milk income in the current lactation. In Irish dairy herds a reduction in milk yield of 1 259.3 kg per lactation could be observed in cows with clinical JD compared to control animals between the years 1994 and 2004 (Richardson and More, 2009). Also the average cull price of animals with clinical signs was 516 € less, than for culled animals without clinical signs. However, to estimate the true economic costs for a MAP infection is quite difficult, because of uncertain estimated prevalences and the number of subclinical infections.

1.1.4 Eradication strategies

Therapy and vaccination

Drugs against MAP infection were tested in vitro and in vivo (reviewed by Cocito *et al*., 1994). Thereby, most of the antimicrobacterial agents, including antibiotics and antimetabolites, inhibited the in vitro growth of MAP. But, the in vivo results showed some discrepancies. Antibiotics could dilute the clinical symptoms but could not eliminate the infection, shown by positive fecal culture and biopsy results. To conclude, there is no effective therapy against MAP infection.

The vaccination of cattle against MAP hides some problems. On the one hand, since vaccination does not provide complete immunity, the vaccinated animals cannot be named as free of MAP infection (Rossiter and Burhans, 1996). On the other hand, immune based diagnostic tests for MAP, which are often used in control programs, and the immune response to vaccination interfere with each other, and lead to false-positive test results (Köhler *et al*., 2001; Muskens *et al*., 2002). A third problem is the strong antigenic relationship between mycobacterium species, leading to a possible interference with the diagnosis of bovine tuberculosis, caused by *Mycobacterium bovis* (*M. bovis*). These leads to complications of bovine tuberculosis detection in herds vaccinated against MAP (Köhler *et al*., 2001; Thomsen *et al*., 2012). In Germany no licensed vaccine is on the market (Paul-Ehrlich-Institut, 2013).

Management

Because of the lack of therapy and vaccination, management is one of the main points in the control of ParaTB. There are two management strategies to control ParaTB: keep free herds free and control or eradicate MAP in infected herds.

On herd level especially proper hygiene and biosecurity measures are important, not only specifically to control ParaTB but also to control other pathogens such as BVD Virus, Salmonella, *Bovine* herpes virus (BHV) and mastitis pathogens (Sweeney *at al*., 2012). Recommendations to prevent MAP infection into MAP-free herds are summarized by Sweeney *et al*. (2012). Mainly, the introduction of the agent should be avoided, for example by buying animals only with known ParaTB status, not participating in auctions or shows where contact to animals with unknown status is possible, not using potential contaminated equipment, control and restrict the herd-contact for veterinarians and other professionals, as well as for third-persons.

Weather the herd is already infected the attention should be turned especially on the calves to prevent new infections, because they are the most susceptible animals. New born calves and young livestock should be kept away from contact with fecal matter from adult cattle (via feed, water, dust, or other environmental sources) or other potential contaminated sources (Doré *et al*., 2012; Sweeny *et al*., 2012). Therefore, the most efficient strategy is the separation of newborn calves from cows, also spatial separation (preferable in separate barns) of calving-raising areas from adult animals, and separate used equipment for the calves (Doré *et al*., 2012). Specific recommendations for dairy cows (e.g. only to feed colostrums from MAP free cows) and beef cow-calves are summarized by Sweeny *et al*. (2012). Another aspect in MAP infected herds is the handling of infected animals. Depending on the goal of the farm or the control program (reducing the prevalence or eradication of the agent) the test and cull or test and management strategy is used.

Control programs

Within the European Union 86 196 Million cattle were counted in 2012, with 12 507 Million from Germany (Statista, 2013). The different frame conditions for dairy and cattle

production, as well as the differences in legislation and administration between the countries lead to diverse control efforts and programs for MAP.

For example, Sweden which is deemed to be almost free of the disease, performs a rigorous mandatory control program with a stamping out policy (Khol and Baumgartner, 2012). In Austria ELISA and fecal culture were used for ParaTB diagnostic in animals with clinical signs. The positive animals have to be separated. Within three days positive tested animals have to be culled, the meat has to be disposed, and hygienic precautions to prevent further spreading of the disease have to be performed on the farm. The government compensates for the culled animals (Khol *et al*., 2007). The Netherlands established a voluntary control program in 2006 (Weber and Schaik, 2007). At this juncture, all lactating cows were tested by milk ELISA, and cattle over 3 years of age by serum ELISA for MAP antibodies. Participating herds are assigned as "Status A" (without positive ELISA test result), as "Status B" (positive animals have been removed) and as "Status C" (positive animals remaining in the herd; Weber *et al*., 2009). 85% of the Dutch Dairy herds are participating, since 2008 the first tests are paid by the Dutch Dairy Board (Weber *et al*., 2009). In Germany cases of ParaTB have to be reported and registered, without consequences for the reported animals or herd (BGBl, 2013). Guidelines, which summarized suggestion for hygienic precautions and control programs for MAP, with the aim to reduce prevalence, clinical science, and further distribution of the disease are published by the Ministry of Food, Agriculture and Consumer Protection in 2005 (BMVEL, 2005). A uniform program in Germany doesn't exist, but some of the federal states established voluntary control programs, e.g. Thuringia, Saarland, North Rhine-Westphalia, Lower Saxony and the federal state of Brandenburg (Khol and Baumgartner, 2012).

1.2 Genetics of host susceptibility to paratuberculosis

For a better understanding of the mechanism of ParaTB and to improve programs for the reduction of MAP infection suceptibility, it is important to get a better knowledge concerning genetic variation of MAP infection. Also, a sustainable and efficient alternative solution is necessary to the lack of therapy and vaccination. Thereby, creating a more

resistant population through breeding programs should not be considered a complete solution for ParaTB but rather one tool that could potentially be used to prevent or reduce incidence of infection.

1.2.1 Estimates of heritability for MAP infection

The estimated heritability for MAP susceptibility in dairy cattle ranges from 0.06 to 0.18 summarized in Table 3 including the used evaluation models and the methods of phenotyping for MAP infection status (Koets *et al*., 2000; Mortensen *et al*., 2004; Gonda *et al*., 2006; Hinger *et al*., 2008; Attalla *et al*., 2010, van Hulzen *et al*., 2011).

The various diagnostic methods used for phenotyping, the divergent statistical approaches (linear- or threshold model, animal- or sire model) and the variances in the sample size from 3 020 (Koets *et al*., 2000) to 684 364 animals (van Hulzen *et al*., 2011) are reasons for the different heritabilities. In the most studies the infection has been determined by ELISA with an average estimated heritability of 0.09 – 0.10 (Mortensen *et al*., 2004; Gonda *et al*., 2006; Hinger *et al*., 2008; Attalla *et al*., 2010; van Hulzen *et al*., 2011). On the opposite the estimated heritability for an MAP infection defined by culture of the feces is 0.15 and therefore higher (Gonda *et al*., 2006).

Table 3: Estimated heritability for infection with *Mycobacterium avium* ssp. *paratuberculosis* in Holstein Friesian (HF) and other dairy cattle.

N =	Phenotype		Model	h^2 estimated	Reference
3 020 unknown breed	microbiological and histological	All	Threshold[§]	0.06 (±0.037)	Koets *et al*. (2000)
		Vaccinated only	Threshold[§]	0.09 (±0.050)	
		Non-vaccinated	Threshold[§]	< 0.01	
		Dam, daughter	Threshold[§]	0.08 (±0.095)	
11 535 HF	ELISA milk		Linear	0.102	Mortensen *et al.* (2004)
			Linear [§]	0.101	
			Linear[*]	0.091	
4 603 HF	Fecal culture		Threshold[*]	0.153 (±0.115)	Gonda *et al*. (2006)
	Fecal culture		Bivariate[2]	0.125 (±0.096)	
	ELISA		Linear[*]	0.159 (±0.090)	
	ELISA		Bivariate[2]	0.183 (±0.082)	
	ELISA		Ordered[3]	0.091 (±0.053)	
	Combined[4]		Threshold[*]	0.102 (±0.066)	
4 524 HF	ELISA	Questionable = pos[5]	Threshold[§]	0.062 (±0.025)	Hinger *et al*. (2008)
		Questionable = neg[5]	Threshold[§]	0.052 (±	
		Questionable = pos	Linear[§]	0.120 (±0.024)	
		Questionable = neg	Linear[§]	0.136 (±0.043)	
2 084 HF		Optical density value	Linear[§]	0.102 (±0.043)	
50 493 HF	ELISA milk		Threshold[§]	0.065	Attalla *et al*. (2010)
			Linear[§]	0.080	
Including		Including maternal	Threshold[§]	0.095	
Including		Including maternal	Linear[§]	0.075	
684 364 HF	ELISA milk		Threshold#	0.031 (±0.002)	van Hulzen *et al*. (2011)
265 290 HF		Herds with ≥2 pos.	Threshold#	0.041 (±0.004)	
104 382 HF		Herd prevalence ≥5%	Threshold[#]	0.060 (±0.006)	
28 916 HF		Herd prevalence ≥10%	Threshold[#]	0.097 (±0.014)	

[§]animal model; [*]sire model; [#]sire maternal grand sire model

[1]with Optical density values and daily milk yield as dependent variables

[2]with ELISA and fecal culture as dependent variables

[3]ordered based on ELISA, Optical density values for the serum (positive control and negative control) were converted to sample-to-positive ratios.

[4]MAP positive if positive to either ELISA or fecal culture

[5]pos = positive, neg = negative

1.2.2 Candidate gene studies

Candidate genes are genes which influence directly or indirectly the phenotypic characterization of the investigated trait because of the position (positional candidate genes) on the chromosome identified by Quantitative trait loci (QTL) studies, and / or because of their biological (functional candidate gene) function (Zhu and Zhao, 2007).

Several studies describing potentially functional candidate genes for an infection with MAP are published (Purdie *et al*., 2011). In particular, the following genes are of interest: *solute carrier family 11 member 1* (*SLC11A1*; Pinedo *et al*., 2009a; Ruiz-Larrañaga *et al*., 2010a), *Toll-like receptors* (*TLRs*; Hinger, 2009; Mucha *et al*., 2009; Pinedo *et al*., 2009a), *Interleukin* (*IL*), especially *IL10* (Verschoor *et al*., 2010), *interferon-gamma* (*IFN*-γ; Pinedo *et al*., 2009b) and the *Nucleotide Oligomerization Domain 2* (*NOD2*; Hinger, 2009; Pinedo *et al*., 2009c; Ruiz-Larrañaga *et al*., 2010b).

SLC11A1, formerly known as the natural resistance-associated macrophage protein 1 (*NRAMP1*; Pinedo *et al*., 2009a; Ruiz-Larrañaga *et al*., 2010a), is part of the innate immune response and plays an important role in preventing bacterial growth in macrophages during the initial phase of infection. Allelic variants of the gene are associated with the natural resistance against pathogens like mycobacterial species, *Salmonella thyphimurium*, and *Leishmania donovani* in mice (Vidal *et al*., 1993; Pinedo *et al*., 2009a).

In mammals 13 different *TLRs* have been identified so fare (Gerold *et al*., 2007). The function of the *TLRs* is the recognition of the so-called pathogen associated molecular patterns (PAMPs), molecules of foreign proteins, the regulation of the innate and the adaptive immunity (Takeda *et al*., 2003). Especially, mutations in *TLR1*, *TLR2*, and *TLR4* are associated with MAP infection in cattle (Mucha *et al*., 2009). TLR4 identifies especially cell wall components of gram-negative bacteria, whereas TLR1 and TLR2 recognize a variety of components from different microorganisms, amongst others lipoproteins from mycobacteria (Takeda *et al*., 2003). The *TLR4* as candidate gene for MAP susceptibility in cattle was also of interest in studies of Hinger (2009) and Pinedo *et al*. (2009a). However, no associations of polymorphisms in the *TLR4* gene and MAP infection were found.

Interleukins are important in the signaling cascade of the immune response to a pathogen, whereas IFN-γ innate the host response to numerous mycobacterial infections. Associations between polymorphisms in *IL10* (Verschoor *et al*., 2010) and *IFN*-γ (Pinedo *et al*., 2009b) and MAP susceptibility were detected.

NOD2 is an intracellular receptor, which recognizes mycobacterial cell wall component (Oh *et al*., 2005) and is part in the activation of the NF-κB- (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) proinflammatory signaling pathway (Ogura *et al*., 2001).

Polymorphisms in the *NOD2*, formerly known as *Caspase Recruitment Domain*, *Member* 15 (*CARD15*), were associated with a higher risk to CD in humans (Hugot *et al*., 2001; Ogura *et al*., 2001). Because of the similarity between Crohns's disease and Johne's disease, *NOD2* is an interesting candidate gene for ParaTB infection in bovine. Moreover, the gene is well characterized and numerous polymorphisms have been identified in coding and non-coding regions in cattle (Taylor *et al*., 2006). So far three different polymorphisms are described with an association to MAP infection: two in Holsteins (Ruiz-Larrañaga *et al*., 2010b; Hinger, 2009), and one in Brahma-Angus crosses (Pinedo *et al*., 2009c).

1.2.3 Whole genome scan

The first whole genome scan study for MAP infection in cattle was done in 2007 by Gonda *et al*. Therefore, 159 microsatellites in US Holstein half-sib families were analyzed. The MAP status of the animals were determined by ELISA and /or fecal culture. The result of the study is a putative QTL affecting susceptibility to MAP infection on bovine chromosome (BTA) 20.

The successes of classical QTL analysis are limited especially in complex traits and / or in traits with low heritability. The limitations are due to the imprecisely mapping of QTL (with confidence intervals spanning a region of 50 cM) and because of the fact that linkage phases varied between families. Consequently, several thousand animals have to be included into the analysis to determine small QTL effects leading to high typing costs (Rothschild *et al*., 2007; Goddard *et al*., 2010). To overcome this limitation, the genome wide association (GWA) study is one of the most promising approaches in agriculture for the improvement of economically important traits. High-density single nucleotide polymorphism (SNP) arrays become feasible in domestic animals as a result of the advances in whole-genome sequencing and the development in "next-generation" sequencing technologies (Fan *et al*., 2010). Moreover, in the last decade the costs for these techniques dropped down and make SNP arrays as a state of art tool for genetics and genomics. The most fundamental change for breeding and genetics in agriculture as a direct result of the application of SNP array seems to be the genomic selection (Fan *et al*., 2010).

The first GWA study relating to MAP susceptibility utilizing SNP arrays was published by Settles *et al*. (2009). 218 US Holsteins tested by MAP culture from feces and necropsy tissue, were genotyped with the Illumina BovineSNP50 BeadChip. An association between the MAP status of the cows and genomic regions on BTA 3 and BTA 9 was reported. Since then, four other GWA studies were published identifying various genomic regions associated with the MAP infection status of the animals typed by different test methods: on BTA 9, 11, and 12 in Italian Holsteins tested by serum ELISA (Minozzi *et al*., 2010), on BTA 1, 5, 6, 7, 10, and 11 in US Holsteins tested by milk or serum ELISA (Pant *et al*., 2010), on BTA 3, 6, 14, 16, 17, 20, 23, 24, and 26 in US Holsteins tested by fecal culture or ELISA (Kirkpatrick *et al*., 2011), and on BTA 4, 15 and 28 in Dutch Holsteins tested by milk ELISA (van Hulzen *et al*., 2012). Additional 16 putative SNPs associated with MAP infection status determined by serum ELISA or tissue culture were identified distributed over the chromosomes 1, 6, 7, 9, 12, 13, 15, 16, 21, 22, 23, and 25 within a Meta-analysis by Minozzi *et al.* (2012) of two GWA studies.

1.3 Aims and structure of the thesis

Aim of the thesis

Because of the lack of treatment and vaccination against Johne's disease, time and cost consuming management strategies are so fare the only eradication strategies against MAP infections and therefore genetic selection comes to the fore. The bases for selection are an adequate heritability, and /or the association of single genes or whole genomic regions to MAP infection in animals and a reliable phenotype assessment of the disease status of animals. Different studies, described in the introduction of the thesis, addressed these points. Due to the problem of standardized, comprehensive, fast, and competitive test methods for phenotyping the MAP status of the cows, most studies deal with milk or serum ELISA test results or a mix of different test methods, while fecal culture is considered as "gold standard". Therefore, the aims of the study were to:

I. estimate the heritability for MAP infection tested by fecal culture on a large dataset,

II. analyze the effect of the ParaTB status of fecal culture tested cows on milk production traits and calving interval,
III. analyze the multiple mentioned candidate gene *NOD2* and the association to the MAP status of the cows tested with fecal culture,
IV. identify further or to confirm so fare identified regions of interests in the bovine genome associated with the MAP status of the animals, and
V. compare different ELISA test methods and the fecal culture regarding the above mentioned goals.

Structure of the thesis

The thesis is divided into four main parts.

Study 1

The goal of the first study was to estimate the heritability in German Holstein population for MAP infection tested with fecal culture, to validate the hypothesis that fecal culture is a more useful tool as selection criteria against MAP than serum / milk ELISA. Therefore, the data of 11 285 cows with full pedigree information from 15 farms in Thuringia were used. Further details about the statistical analysis and the used models as well as the results followed by a detailed discussion will be presented in the study in chapter 2:

Küpper, J., Brandt, H., Donat, K. & Erhardt, G. (2012). Heritability estimates for *Mycobacterium avium* subspecies *paratuberculosis* status of German Holstein cows tested by fecal culture. J. Dairy Sci. 95: 2734-2739.

Study 2

In the second part of the thesis the hypothesis of economic losses due to MAP infection were checked. For the analyses the 305-day milk yield, milk fat and milk protein from 9 367 German Holstein herd book cows were tested by fecal culture and serum ELISA, as well as milk kg per day of live and the calving interval as parameter for reproduction. The data structure, analysis and results are discussed in the study illustrated in chapter 3:

Küpper, J., Brandt, H., Donat, K. & Erhardt, G. (2013). Associations between paratuberculosis status and milk production traits in Holstein cattle under consideration of

interaction effects between test result and farm and lactation number. Arch. Tierz., doi: 10.7482/0003-9438-56-094.

Study 3

The third study deals with the analysis of the putative candidate gene *NOD2*. With the aid of a case-control study, associations between SNPs in the candidate gene and the MAP infection status tested by fecal culture and serum ELISA of the animals were performed. For the case-control study 162 fecal culture positive animals and 162 fecal culture negative animals with the same father, from the same farm and in the same age were selected. The publicaion in chapter 4 compromises the topic of the candidate gene analysis of the *NOD2* with details of the experiment and the results concluded by the discussion:

Küpper, J., Brandt, H. & Erhardt, G. (2014). Genetic association between *NOD2* polymorphisms and infection status by *Mycobacterium avium* ssp. *paratuberculosis* in German Holstein cattle. Anim. Genet. 45: 114–116.

Study 4

A case-control whole genome association study was used for the fourth part of the study to identify genetic markers and genomic regions associated with the risk of MAP infection. For the case-control study 152 fecal culture positive animals and 153 fecal culture negative animals (same father, same farm and same age) were genotyped with the Illumina Bovine SNP50BeadChip. To compare the results of different test methods, all cows were tested by serum ELISA. The experimental design, the data analysis and the results including discussion are given in chapter 5 which includes the following study:

Küpper, J., Brandt, H., Donat, K. & Erhardt, G. (2013). Phenotype definition is a main point in genome-wide association studies for bovine *Mycobacterium avium* ssp. *paratuberculosis* infection status. Accepted for publication in Animal: April 03, 2014.

2. HERITABILITY ESTIMATES FOR *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* STATUS OF GERMAN HOLSTEIN COWS TESTED BY FECAL CULTURE

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ABSTRACT

The objective of this study was to estimate genetic manifestation of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) infection in German Holstein cows. Incorporated into this study were 11,285 German Holstein herd book cows classified as MAP-positive and MAP-negative animals using fecal culture results and originating from 15 farms in Thuringia, Germany involved in a paratuberculosis voluntary control program from 2008 to 2009. The frequency of MAP-positive animals per farm ranged from 2.7 to 67.6%. The fixed effects of farm and lactation number had a highly significant effect on MAP status. An increase in the frequency of positive animals from the first to the third lactation could be observed. Threshold animal and sire models with sire relationship were used as statistical models to estimate genetic parameters. Heritability estimates of fecal culture varied from 0.157 to 0.228. To analyze the effect of prevalence on genetic parameter estimates, the total data set was divided into 2 subsets of data into farms with prevalence rates below 10% and those above 10%. The data set with prevalence above 10% show higher heritability estimates in both models compared with the data set with prevalence below 10%. For all data sets, the sire model shows higher heritabilities than the equivalent animal model. This study demonstrates that genetic variation exists in dairy cattle for paratuberculosis infection susceptibility and furthermore, leads to the conclusion that MAP detection by fecal culture shows a higher genetic background than ELISA test results. In conclusion, fecal culture seems to be a better trait to control the disease, as well as an appropriate feature for further genomic analyses to detect MAP-associated chromosome regions.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, heritability, fecal culture

INTRODUCTION

Paratuberculosis (ParaTB) or Johne's disease (JD) is a widespread chronic enteritis of ruminants caused by the agent *Mycobacterium avium* ssp. *paratuberculosis* (**MAP**). The animals are infected as calves but due to the characteristically long incubation time of up to 15 yr, they initially show no clinical symptoms (Chiodini et al., 1984). The first period of

illness is characterized by intense, intermittent, and chronic diarrhea, causing weight loss to the point of death (Chiodini et al., 1984). High economic losses are incurred through diminished milk and fattening performance, decreased fertility, as well as costs associated with the management of infected animals (McKenna et al., 2006).

Strategies decreasing the incidence of paratuberculosis so far involve the detection and culling of infected animals as well as other management strategies to avoid further dispersion of MAP. The diagnosis of MAP-infected animals can be carried out directly via fecal PCR, using fecal or tissue culture for agent detection, or indirectly through MAP antibody detection in blood or milk with commercial ELISA tests (Harris and Barletta, 2001). The problem with all of these tests is the limited sensitivity or specificity to detect infected animals at an early stage of infection (Nielsen and Toft, 2008). To improve the accuracy of diagnosis, it is possible and advisable to combine different detection methods (Böttcher and Gangl, 2004).

Due to long incubation times, subclinical phases (Chiodini et al., 1984) and problematic diagnostics, a definite statement on MAP occurrence is hardly possible. The highest MAP prevalence of single herds is based on extrapolations of single regions of the country (Dreier et al., 2006). For example, herd prevalence rates in Europe range from 6.9% in Austria (Dreier et al., 2006) to 54.7% in the Netherlands (Muskens et al., 2000). In the United States, prevalence was estimated to be 87.5% (Wells et al., 2002) and for Canada between 34.0 and 58.0% (Hendrick et al., 2005). Within-herd prevalences are found in a wide range from below 1% (Donat et al., 2011) to more than 30% (Gasteiner et al., 1999; Hinger et al., 2008).

Heritabilities for MAP status were first estimated in the Netherlands by Koets et al. (2000). Further studies estimated heritabilities between 0.031 and 0.159 (Mortensen et al., 2004; Gonda et al., 2006; Hinger et al., 2008; Attalla et al., 2010; van Hulzen et al., 2011). Differences in estimated heritability results could be caused by the various diagnostic methods used to detect MAP, divergent statistical approaches estimating variance components, sire models versus animal models, or threshold versus linear models, as well as different prevalence rates in the data as shown by van Hulzen et al. (2011). Koets et al. (2000) classified the positive animals on microbiological and histopathological analyses after slaughtering, whereas most other studies used ELISA to determine specific antibodies

against MAP in milk or blood (Mortensen et al., 2004; Gonda et al., 2006; Hinger et al., 2008; van Hulzen et al., 2011). A third factor influencing the heritability estimates could be variances in sample sizes from 684,364 animals (van Hulzen et al., 2011) to 3,020 animals (Koets et al., 2000).

In general, the sensitivity of ELISA tests is low, meaning that infected animals do not always show a positive ELISA test result. Therefore, fecal culture is considered as the most sensitive method for detecting MAP in cattle (Collins et al., 2006) and is deemed the gold standard (Chacon et al., 2004). So far only 1 study on heritability estimates based on fecal culture has been published (Gonda et al., 2006). These estimates are based on a data set from a QTL study with data from 12 sires only, which hardly can be seen as a random sample of sires. The objective of this study was to estimate genetic variation and heritability for paratuberculosis infection in a large data set of German Holstein cows classified as MAP-positive and MAP-negative animals using fecal cultural results.

MATERIALS AND METHODS

Data Set

A data set of 11,285 German Holstein herd book cows originating from 15 farms was available. All animals were routinely screened for MAP status in 2008 and 2009 as part of a voluntary program in Thuringia in Germany (Donat, 2009) by fecal culture (AVID, 2007), whereas a total of 1,218 animals (10.79%) tested positive and 10,067 MAP negative. For all animals only a single test result was available.

The 11,285 cows descended from a total of 869 sires with an average of 12.8 daughters per sire, but with large differences in the number of daughters among the sires. Three hundred and ninety-two sires had more than 4 daughters, with an average of 25.8 daughters per sire. The maximum was 445 daughters per sire. The distribution of sires and number of daughters is shown in Table 1. For further analyses, the total data set was divided into 2 subsets with data from farms with MAP prevalence rates below 10% and above 10%. In the first subset (<10% prevalence), 9 farms were included with a total of 654 sires and an average number of

daughters per sire of 10.3. In the second subset, 6 farms with a total of 468 sires and an average number of daughters per sire of 9.0 were included. The distribution of sires and number of daughters for these 2 subsets is shown in Table 1.

Table 1. Distribution of number of daughters per sire

Number of daughters	Number of sires		
	Complete data set	Prevalence within herd <10%	Prevalence within herd >10%
1	206	155	131
2	131	109	102
3	77	72	47
4	63	52	30
> 4	392	266	158
Total	869	654	468

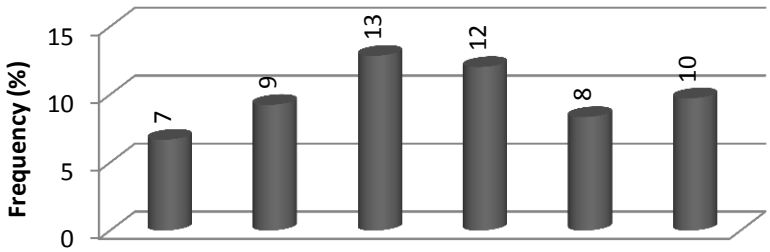
Statistical Analysis

The statistical analyses (SAS GLIMMIX and ASREML; SAS Institute Inc., Cary, NC) were carried out with logistic regression models using the MAP status as a binomial random variable. The logistic models use the logit link function, which is defined by $\ln[p_i/(1 - p_i)]$, where p_i is the probability of an animal to be MAP positive.

The data were analyzed with the GLIMMIX procedure of SAS 9.1 to estimate the significance of fixed effects on the MAP status of cows. Farm and lactation number as age effect were included in the model as fixed effects. All lactation numbers above 5 were combined into 1 class. To estimate the variance components, farm and lactation number as fixed effects and sire as random effect or, alternatively, animal was included in a threshold model (logit function) using the software package ASREML (Gilmour et al., 2009). Heritabilities were calculated using the logit function, which implies a correction of the residual variance by the factor $\pi^2/3$. Within the sire model, the relationship between sires (6,535 animals in total) was included in the model. Within the animal model, the full relationship between all animals (42,195 animals in total) was included in the model. The variance components using

the sire model were estimated including all 869 sires and, alternatively, only the 392 sires with more than 4 daughters in the data set.

One farm showed a very high MAP prevalence of 68%, whereas the other farms showed prevalence rates below 30%. Therefore, the variance components with the sire and the animal models were also estimated excluding the farm with the high prevalence rate. Additionally, the variance components with the sire model and the animal model were also estimated within the 2 subsets of data based on test prevalence: 1) herds with a prevalence <10% and 2) herds with a prevalence >10%. The different models were used to analyze the effects of sire versus animal model as well as different herd prevalence rates on heritability estimates for comparison to Hinger et al. (2008) and to van Hulzen et al. (2011).



Lactation	1	2	3	4	5	>6
Average age (mo)	31	44	58	71	85	105
n	4,471	3,126	1,782	979	450	323

Figure 1. Estimated frequency of fecal tested *Mycobacterium avium ssp. paratuberculosis* (MAP)-positive German Holsteins cows per lactation number and average age of the cows per lactation number.

RESULTS

The fixed effects farm and number of lactation had a high significant effect ($P < 0.001$) on the MAP status. The estimated MAP frequency based on fecal-positive cows within lactation number and the average age within lactation number are summarized in Figure 1. We observed an increase in the frequency of positive animals from the first to the third lactation. Animals in the third and fourth lactation show the highest values with 13 and 12%,

respectively, whereas fecal-positive tested cows with 6 or more lactations showed a frequency of 10% positive animals. The frequency of MAP-positive animals per farm, shown in Figure 2, varied from 2.8% (farm number 6) to 67.6% (farm number 3).

The estimated variance components and heritabilities for both models and all 5 data sets are shown in Table 2. Estimated heritabilities range from 7.8 to 28.3%. The highest heritability, as well as the highest additive genetic variance, was estimated in the sire model on herds with prevalence above 10%. The lowest additive genetic variance was estimated with the animal model within herds with prevalence below 10%. The exclusion of farm number 3 did not show an effect on the residual variance for the animal model, but a small decrease in the residual variance for the sire model. The heritability estimates are, therefore, slightly higher than from the complete data set. The sire models showed higher heritabilities than the equivalent animal models for all data sets. For animal and sire models, the data set with prevalence below 10% showed a much lower heritability than the data set with prevalence above 10%, caused by the much higher estimates of additive genetic variance in both models for the latter data set.

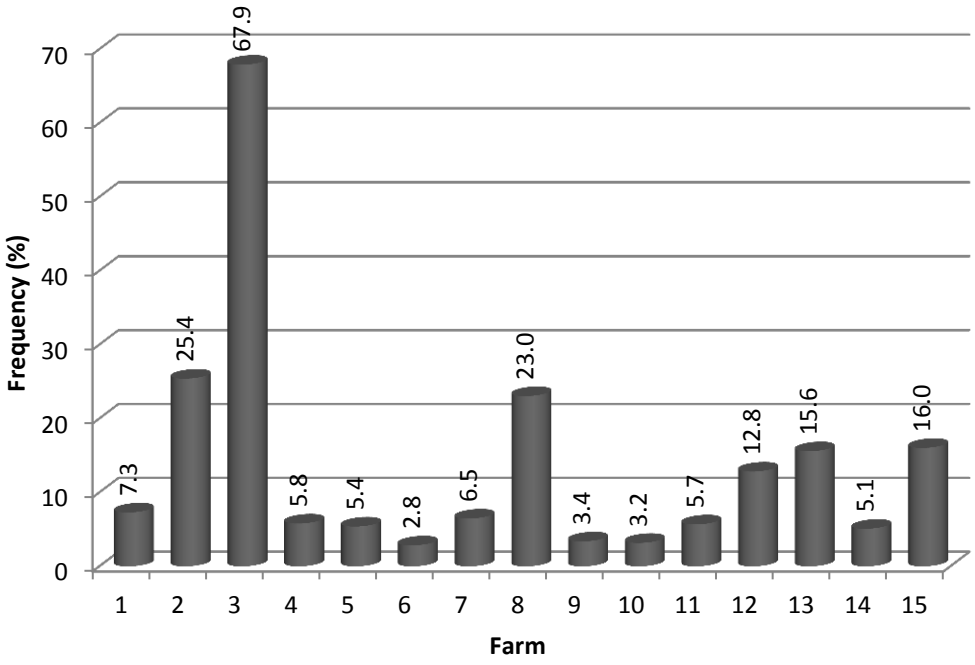


Figure 2. Estimated frequency of fecal-tested *Mycobacterium avium ssp. paratuberculosis* (MAP)-positive German Holsteins cows per farm.

DISCUSSION

The different prevalence rates within farms are most likely a result of the manner and duration of eradication procedures on the one hand or could be caused by different environmental and management factors on the other. Especially the management of replacement as well as the calving and hygiene practices played important roles (Raizman et al., 2004; McKenna et al., 2006; Collins et al., 2010). Farm number 3 with the very high prevalence of almost 68% had not reacted with culling of positive animals so far. The other farms had already been involved in the voluntary surveillance program in Thuringia for a longer period, but before 2008, blood ELISA test results were used to detect positive and negative animals. The change in prevalence over time within farms has not yet been analyzed and, therefore, a possible selection response cannot be quantified.

Table 2. Estimates of heritabilities (h2) with standard errors and additive genetic (VA) and phenotypic variances (VP) for different models and data sets

Data set	n	Model	h2 ± se	VA	VP
1: Complete	11,129	Sire	0.195 ± 0.054	0.675	3.469
		Animal	0.157 ± 0.024	0.613	3.913
2: Sires with > 4 daughters	10,126	Sire	0.175 ± 0.051	0.602	3.451
3: Without farm number 3	10,555	Sire	0.228 ± 0.061	0.799	3.500
		Animal	0.168 ± 0.026	0.666	3.966
4: Prevalence within herd < 10%	6,823	Sire	0.112 ± 0.091	0.378	3.395
		Animal	0.078 ± 0.051	0.278	3.578
5: Prevalence within herd > 10%	4,306	Sire	0.283 ± 0.078	1.006	3.551
		Animal	0.138 ± 0.028	0.771	4.071

Hinger et al. (2008) showed an increase of ELISA-positive tested animals up to an age of 5 yr. This result corresponds to the increase in positive animals up to the third lactation (see Figure 1) observed in this study with an average age of 58 mo. The present results and those from Jubb and Galvin (2000), Dreier et al. (2006), and Hinger et al. (2008) using ELISA tests leads to the conclusion that both methods (fecal culture and ELISA) show the same tendency of MAP prevalence over age groups. This result was not expected, as Eamens et al. (2000)

have shown that fecal culture testing and serology did not show a high correlation. They confirmed only 37% of fecal positive cattle by ELISA testing. For a subgroup of animals from the total data set in this study, both fecal culture as well as ELISA test results are available (publishing in progress). Due to its higher sensitivity, as shown by McKenna et al. (2005) and Köhler et al. (2008), testing by fecal culture results in a higher prevalence then the ELISA test results. Only a very small proportion of fecal-negative tested animals were positive using ELISA tests (<5%), which is a consequence of the limited specificity (McKenna et al., 2005; Köhler et al., 2008) of the ELISA tests. From the development shown above, it could be assumed that infected animals detected by ELISA tests react simultaneously positive in fecal culture.

The estimated heritabilities from the complete data set of the binary criteria fecal positive or negative between 15.7% for the animal and 19.5% for the sire model are in agreement with Gonda et al. (2006). Based on fecal-tested animals, they estimated heritability in US Holsteins to be 15.3%. The heritability based on ELISA tests are between 0.031 and 0.16, depending on ELISA test (milk or blood) and the breed (Mortensen et al., 2004; Gonda et al., 2006; Hinger et al., 2008; Attalla et al., 2010; van Hulzen et al., 2011).

In our study, the estimated heritability using the animal model was lower than the estimated heritability by using the sire model for all data sets, which is in agreement with Hinger et al. (2008). In cases of sires with a small number of daughters, an advantage exists in using an animal model rather than a sire model (Ramirez-Valverde et al., 2001). These results do not support the assumption made by van Hulzen et al. (2011) that an animal model will estimate higher additive genetic variance due to possible intrauterine infections of calves with MAP. Comparing the results from the sire model and the sire model with more than 4 daughters (Table 2) confirmed the results of Ramirez-Valverde et al. (2001). The latter model shows a lower heritability than the other sire model, caused by the lower estimated genetic variance, which is close to the estimated genetic variance of the animal model from the same data set. Farm number 3 with the high prevalence did not affect the phenotypic variance but slightly increased the additive genetic variance estimate. This is not in agreement with the effect of higher heritability estimates from data with increasing within-herd prevalence as found by van Hulzen et al. (2011) and also confirmed in the current study from the 2 subsets of data based on prevalence within farms. The sire and the animal models from the subset with

prevalence within herds below 10% show a much lower additive genetic variance than the data set with prevalence within herds above 10%. This effect could be explained on one hand by a possible selection effect through culling of positive-tested animals. On the other hand, it is well known that the additive genetic variance in the case of a trait influenced by only a single gene with 2 alleles is dependent on the allele frequency, and with decreasing allele frequency, additive genetic variance also decreases. In case the MAP status is affected by only a small number of genes, the decrease in additive genetic variance is expected with decreasing prevalence within herds and also with increasing prevalence above 50%, as farm number 3 shows.

In the case of breeding, it seems advantageous to use fecal diagnostics instead of ELISA tests to detect positive animals because of the higher heritabilities found. Taking into consideration the low conformance between fecal and ELISA test results as shown by Eamens et al. (2000), open questions still exist about culling of animals to achieve MAP-free status success. Also, with respect to future genomic selection, it is necessary to improve the diagnostics for MAP status to achieve high conformance between different test methods.

CONCLUSIONS

The heritability for the susceptibility to MAP estimates of fecal culture results are between 7.8 and 28.3% from sire and animal models and different data sets. The results of the study confirm a low to medium genetic background for susceptibility to paratuberculosis infection in dairy cows. The heritability indicates that fecal culture diagnosis is a useful tool as a selection criterion against Johne's disease to increase resistance in cattle. Moreover, it is useful as a selection criterion in breeding programs as well as for further studies on genomic selection or candidate genes.

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REFERENCES

Attalla, S. A., A. J. Seykora, J. B. Cole, and B. J. Heins. 2010. Genetic parameters of milk ELISA scores. J. Dairy Sci. 93:1729–1735.

AVID (Arbeitskreis Veterinarmedizinische Infektionsdiagnostik). 2007. Kultureller Nachweis von *Mycobacterium avium* ssp. *paratuberculosis* in Kot- oder Organproben. Deutsche Veterinärmedizinische Gesellschaft (DVG) e. V, Gießen, Germany.

Bottcher, J., and A. Gangl. 2004. *Mycobacterium avium* ssp. P*aratuberculosis*—Combined serological testing and classification of individual animals and herds. J. Vet. Med. B Infect. Dis. Vet. Public Health 51:443–448.

Chacon, O., L. E. Bermudez, and R. G. Barletta. 2004. Johne's disease, inflammatory bowel disease, and *Mycobacterium paratuberculosis*. Annu. Rev. Microbiol. 58:329–363.

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

Collins, M. T., V. Eggleston, and E. J. B. Manning. 2010. Successful control of Johne's disease in nine dairy herds: Results of a six-year field trial. J. Dairy Sci. 93:1638–1643.

Collins, M. T., I. A. Gardner, F. B. Garry, A. J. Roussel, and S. J. Wells. 2006. Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. J. Am. Vet. Med. Assoc. 229:1912–1919.

Donat, K. 2009. Umsetzung des Thüringer Paratuberkulose-Landesprogrammes auf der Basis des Erregernachweises—Ein Zwischenbericht. Page 94 in Zusammenfassung 7. Stendaler Symposium. Landesamt für Verbraucherschutz Sachsen-Anhalt, Fachbereich 4 Veterinarmedizin, Stendal, Germany.

Donat, K., U. Schau, and A. Soschinka. 2011. Identification of *Mycobacterium avium* ssp. *paratuberculosis* infected dairy herds by environmental sampling. Berl. Münch. Tierärztl. Wochenschr. 124:360–367.

Dreier, S., J. L. Khol, B. Stein, K. Fuchs, S. Gütler, and W. Baumgartner. 2006. Serological, bacteriological and molecularbiological survey of paratuberculosis (Johne's disease) in Austrian cattle. J. Vet. Med. B Infect. Dis. Vet. Public Health 53:477–481.

Eamens, G. J., R. J. Whittington, I. B. Marsh, M. J. Turner, V. Saunders, P. D. Kemsley, and D. Rayward. 2000. Comparative sensitivity of various faecal culture methods and ELISA in dairy cattle herds with endemic Johne's disease. Vet. Microbiol. 77:357–367.

Gasteiner, J., H. Wenzl, K. Fuchs, U. Jark, and W. Baumgartner. 1999. Serological cross-sectional study of paratuberculosis in cattle in Austria. Zentralbl. Veterinarmed. B 46:457–466.

Gilmour, A. R., B. J. Gogel, B. R. Cullis, and R. Thompson. 2009. ASReml User Guide Release 3.0. VSN International Ltd., Hemel Hempstead, UK.

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

Harris, N. B., and R. G. Barletta. 2001. *Mycobacterium avium* ssp. *paratuberculosis* in veterinary medicine. Clin. Microbiol. Rev. 14:489–512.

Hendrick, S., T. Duffield, K. Leslie, K. Lissemore, M. Archambault, and D. Kelton. 2005. The prevalence of milk and serum antibodies to *Mycobacterium avium* subspecies *paratuberculosis* in dairy herds in Ontario. Can. Vet. J. 46:1126–1129.

Hinger, M., H. Brandt, and G. Erhardt. 2008. Heritability estimates for antibody response to *Mycobacterium avium* subspecies *paratuberculosis* in German Holstein cattle. J. Dairy Sci. 91:3237–3244.

Jubb, T., and J. Galvin. 2000. Herd testing to control bovine Johne's disease. Vet. Microbiol. 77:423–428.

Koets, A. P., G. Adugna, L. L. G. Janss, H. J. van Weering, C. H. J. Kalis, G. H. Wentink, V. P. M. G. Rutten, and Y. H. Schukken. 2000. Genetic variation of susceptibility to

Mycobacterium avium ssp. *paratuberculosis* infection in dairy cattle. J. Dairy Sci. 83:2702–2708.

Köhler, H., B. Burkert, I. Pavlik, R. Diller, L. Geue, F. J. Conraths, and G. Martin. 2008. Evaluation of five ELISA test kits for the measurement of antibodies against *Mycobacterium avium* subspecies *paratuberculosis* in bovine serum. Berl. Münch. Tierärztl. Wochenschr. 121:203–210.

McKenna, S. L. B., G. P. Keefe, H. W. Barkema, and D. C. Sockett. 2005. Evaluation of three ELISAs for *Mycobacterium avium* ssp. *paratuberculosis* using tissue and fecal culture as comparison standards. Vet. Microbiol. 110:105–111.

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

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

Muskens, J., H. W. Barkema, E. Russchen, K. van Maanen, Y. H. Schukken, and D. Bakker. 2000. Prevalence and regional distribution of paratuberculosis in dairy herds in the Netherlands. Vet. Microbiol. 77:253–261.

Nielsen, S. S., and N. Toft. 2008. Ante mortem diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon-γ assay and faecal culture techniques. Vet. Microbiol. 129:217–235.

Raizman, E. A., S. J. Wells, S. M. Godden, R. F. Bey, M. J. Oakes, D. C. Bentley, and K. E. Olsen. 2004. The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. J. Dairy Sci. 87:2959–2966.

Ramirez-Valverde, R., I. Misztal, and J. K. Bertrand. 2001. Comparison of the threshold vs. linear and animal vs. sire models for predicting direct and maternal genetic effects on calving difficulty in beef cattle. J. Anim. Sci. 79:333–338.

van Hulzen, K. J. E., M. Nielen, A. P. Koets, G. de Jong, J. A. M. van Arendonk, and H. C. M. Heuven. 2011. Effect of herd prevalence on heritability estimates of antibody response to *Mycobacterium avium* subspecies *paratuberculosis.* J. Dairy Sci. 94:992–997.

Wells, S. J., R. H. Whitlock, B. A. Wagner, J. Collins, F. Garry, H. Hirst, J. Lawrence, W. J. Saville, and A. L. L. Naugle. 2002. Sensitivity of test strategies used in the Voluntary Johne's Disease Herd Status Program for detection of *Mycobacterium paratuberculosis* infection in dairy cattle herds. J. Am. Vet. Med. Assoc. 220:1053–1057.

3. Associations between paratuberculosis status and milk production traits in Holstein cattle under consideration of interaction effects between test result and farm and lactation

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ABSTRACT

One of the most important factors for economic losses as result of infection with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) is the decrease in milk yield. Different phenotyping methods for MAP lead to inconsistent impact of MAP infection on milk parameters. It was the aim of the study to analyze the effect of the MAP status on milk yield, milk fat and milk protein (in kilograms), as well as on the calving interval and milk kilograms per day of life in German Holstein cattle. A dataset of 9,367 fecal culture tested animals from 14 farms in Thuringia (Germany) were available. The MAP status of the animals affected milk yield and the milk parameters. Beside the effects of farm, lactation number and MAP status, the interaction between MAP status and farm on the milk parameters was significant. The latter result is a possible explanation for the inconsistent results from recent studies. For milk kg per day of life, the interaction between lactation number and MAP status also showed significance.

Keywords: *Mycobacterium avium* ssp. *paratuberculosis*, milk parameters, fecal culture, MAP

INTRODUCTION

Paratuberculosis (ParaTB), also known as Johne's disease, caused by the facultative pathogen M*ycobacterium avium* ssp. *paratuberculosis* (MAP) is a chronic disease of ruminants. The infection of cows with MAP takes place as calves and the infection of a mature animal is rare (Windsor and Whittington, 2009). Whitlock and Buergelt (1996) classified the status of infection with MAP into four stages: first as "silent" infection, second as subclinical disease, third as clinical disease, and fourth as advanced clinical disease. In the first stage animals up to two years of age are infected with MAP. In this early phase of disease there is no apparent evidence of infection and the detection of MAP is not possible by ELISA nor by fecal culture. In the subclinical stage animals show no typical signs of ParaTB but can be susceptible to other diseases. These cows may have detectable antibodies and infection in a small number of animals may be detected by fecal culture, whereas the majority of infected animals in this phase are undetectable. The third stage is characterized by a long incubation time (2-10 years). In this stage typical clinical signs like diarrhea and

weight loss appear. Most animals are culled before stage four, which is characterized by increasingly lethargy, emaciation and profuse diarrhea up to the point of death. As a result of the infection reduced fertility and a reduced milk production in dairy cattle is observed (McKenna *et al*., 2006). Fecal culture as well as available ELISA tests in serum and milk have a low sensitivity with 60 % and respectively 30 % (Collins *et al*., 2006). This leads to a high amount of undetected infected cows.

Recent prevalence studies reviewed by Tiwari *et al*. (2006) mention herd prevalence rates for Canadian regions between 33.3 % and 74.0 %, for US states between 41 % and 66 % and for European countries between 7 % for Austria and 54 % for the Netherlands. The apparent prevalence rates varied because of the different tests and study designs which are used in these studies (Nielsen and Toft, 2008).

Many studies deal with the resulting economic losses of the disease. Factors like permanent culling to decrease the risk of infection, reduced fertility and higher feed intake are involved in the amount of economic losses (Raizman *et al*., 2009). Estimated losses for the US dairy industry were around US$ 200 to US$ 250 million annually (Ott *et al*., 1999). Chi *et al*. (2002) estimated total costs for an average infected 50 cow herd of 2,472 US$/year. One of the important factors for economic losses is the decrease in milk yield. The deficit of milk income in the current lactation is estimated of 276 $ for fecal positive cows vs. negative ones (Raizman *et al*., 2009). More and more studies deal with the context of lactation performances and ParaTB status in cows (Lombard *et al*., 2005; Gonda *et al*., 2007; Raizman *et al*., 2007; 2009; Sorge *et al*., 2010). In all studies the milk yield of MAP positive cows was reduced, but the amount differs between 212 kg (Tiwari *et al*., 2007) and 1803 kg (Raizman *et al*. 2009) per 305-day lactation. In the majority of these studies the ParaTB status of the animals is detected either by fecal culture (Raizman *et al*., 2007; 2009) or by ELISA (Nielsen *et al*., 2002; Sorge *et al*., 2011) or with both (Gonda *et al*., 2007).

The effect of the MAP infection on milk protein and milk fat content is unclear. Some studies found a decrease in milk-fat and -protein content in infected animals (Benedictus *et al*., 1987) whereas other studies found no associations (Nordlund *et al*., 1996; Lombard *et al*., 2005). Also results about other performance traits, such as reproduction or somatic cell score are inconsistent. One main reason for the inconsistency of results from the literature

could be the fact that different definitions of MAP-positive and MAP-negative animals are used, and the fact that in some studies negative and positive animals included are not from the same farm.

Literature shows different definitions of MAP-positive animals based on different tests for MAP as well as the resulting differences in milk production traits between MAP-positive and MAP-negative animals. Thus, this study aimed to further clarify the impact on MAP on production traits by analyzing the interaction between farm effects and the MAP-status of animals.

So far the latter aspect has not been discussed in published literature but may be a possible explanation for the varying results.

MATERIAL AND METHODS

9,367 German Holstein cows from 14 farms in Thuringia all routinely tested for MAP by fecal culture (Donat *et al*., 2012) were analyzed for the study. This data represents nearly all female animals older than 24 month between 2007 and 2009 of these farms. For all animals full pedigree information and milk recording traits were available. All herds included in this study joined the "Paratuberculosis Control Program in Thuringian Cattle Herds" which is organized by the Thuringian Animal Health Service. The program includes, among other measures, annual whole herd screenings (cows > 24 month) for MAP by fecal culture. If an animal was detected as MAP positive, it was not retested the next year, and culling is recommended as outlined by Sweeney *et al*. (2012).

The association between the paratuberculosis status and the different milk performance traits in the actual lactation in which the cow was tested (milk kilograms, fat in kg, as well as protein in kg, all parameters for 305-days) was analyzed using the software system SAS (SAS 9.1, Institute Inc., Cary, USA). For the GLM procedure the following linear model was used:

$$Y_{ijkl} = \mu + f_i + l_j + t_k + (f \times t)_{ik} + (l \times t)_{jk} + e_{ijkl}$$

with:

Y_{ijkl} = phenotypic observation of cow ijkl

μ = constant mean

f_i = fixed effect of farm i (i = 1-14)

l_j = fixed effect of lactation number j (j = 1, 2, 3, ≥4)

t_k = fixed effect of test result k (k = positive or negative)

$(f \times t)_{ik}$ = fixed interaction of farm by test result

$(l \times t)_{jk}$ = fixed interaction of lactation number by test result

e_{ijkl} = random residual effect.

Additionally, we analyzed the differences between positively and negatively tested animals in milk kg per day of life and calving interval in days using all available lactations. In total for the tested cows data of 21.887 lactations are available.

For these traits in the model the effects of farm, lactation number, fecal test result and the interaction between farm and fecal test result as well as the interaction between lactation number and fecal test result were included as fixed effects and the cow as random effect. For the analysis of the calving interval we excluded the first lactations, so 12.933 observations remained for the analysis.

RESULTS

MAP-tests – milk parameters

From the 9,367 animals 1,136 cows are positive and 8,231 animals are negative for MAP tested by fecal culture. The prevalence in the herds ranges between 3.2 % and 64.4 %. Eight farms shows prevalence rates <10 %, three farms between 10 % and 20 %, two farms between 20 % and 30 %, and one farm with 64.4 %. Table 1 shows the animals with full pedigree information and milk recording data grouped by number of lactation and the test results.

Table 1: Distribution of animals per number of lactation, tested for MAP by fecal culture.

Lactation number	MAP-test result	Fecal culture
1	neg pos	3,482 411
2	neg pos	2,322 343
3	neg pos	1,235 207
≥4	neg pos	1,192 175
n= n=	neg pos	8,231 1,136
n=		9,367

The average milk yield (305-days) over all farms is 8,919 kg with 4.03 % fat and 3.36 % protein. The farm with the lowest milk yield has an average of 8,138 kg and the farm with the highest milk yield of 10,428 kg. As shown in table 2 the differences for the 305-day milk, fat and protein yield in kg between the positive and the negative animals are significant. The table also shows that the parameters “farm” and “lactation number” have a significant influence on the milk parameters.

Table 2: Significances of the influence of MAP status, farm, lactation number and interaction effects on milk in kg, fat in kg and protein in kg of the 305-day lactation and milk per day of live and calving intervall.

Parameter	milk	fat	protein	milk /day of live	calving interval
farm	***	***	***	***	***
lactation number	***	***	***	***	***
fecal culture	**	***	***	***	ns
farm x fecal culture	***	**	***	***	ns
lactation number x fecal culture	**	**	***	***	ns

* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

Animals which tested positively for MAP have a lower 305-day milk yield in kg (figure 1). The average difference is 225 kg milk for 305-day milk yield. Also the fat and protein yields in kg for the 305-day lactation (figure 1) are higher in negative animals, than in positive ones.

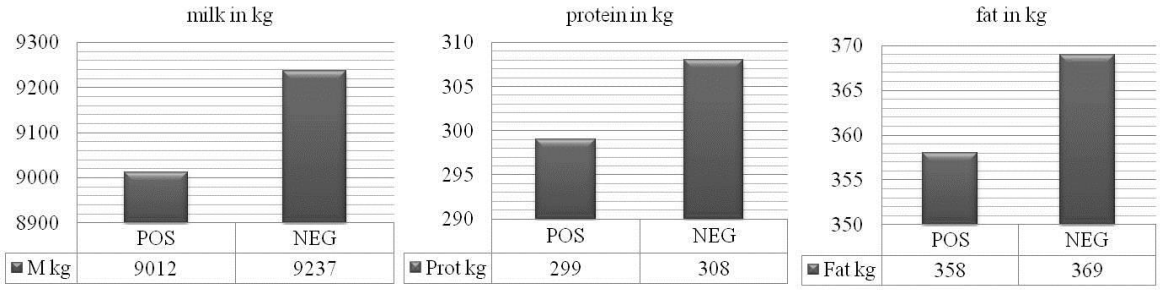

Figure 1: LS means for positive and negative fecal culture tested cows (n = 9,367) for the average 305-day milk yield, fat and protein in kg.

Calving interval

The effects of farm and lactation number show a significant influence on the calving interval (table 2). The negative tested animals show an insignificant two day shorter calving interval in comparison to the positive tested animals. Neither the interactions between fecal test result and farm or lactation number, respectively, show a significant influence on calving interval.

Milk kg per day of live

The fixed effects farm, number of lactation, and fecal culture test results, as well as the interaction between fecal culture test result and farm and lactation number respectively show a high significant influence on the parameter milk kg per day of life (table 2). Figure 2 shows the influence of lactation number on milk kg per day of life for positive and negative tested animals within number of lactation. With increasing lactation number an increasing difference between positive and negative tested animals is observed ranging from 0.02 to 0.81 kg per day of life.

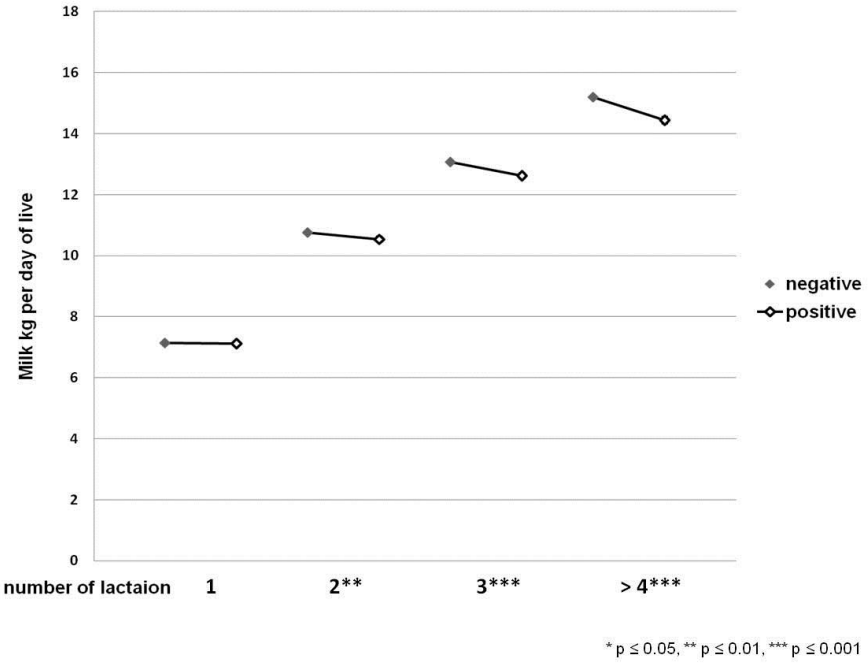

Figure 2: LS means for the milk per day of live in kg and number of lactation in fecal culture tested animals.

Interaction between farm and fecal cultural test result

The significant interaction between farm and fecal cultural test results (table 2) for 305-day milk yield are shown in figure 3. In the majority of farms the negative tested animals show higher milk yield than the positive tested animals. In four of the 14 farms this difference is significant. The differences range between 220 and 904 kg of milk. In three of the 14 farms the positive tested cows show a higher 305-day milk yield ranging between 166 and 445 kg. In two farms the difference between positive and negative tested cows is very small.

As consequence of the interaction component for milk lactation yield and the differences in calving interval as described before a highly significant interaction between farm and test result is also observed for kg of milk per day of life (figure 4). The lower yield of milk per day of life for the positive tested animals is caused by milk reduction, higher first calving age and longer calving interval as shown in farms 8 and 13 especially. In contrast to that in farm 3 the lower kg of milk per day of life for the positive tested animals is only an effect of a very high milk reduction of 904 kg in positive tested animals.

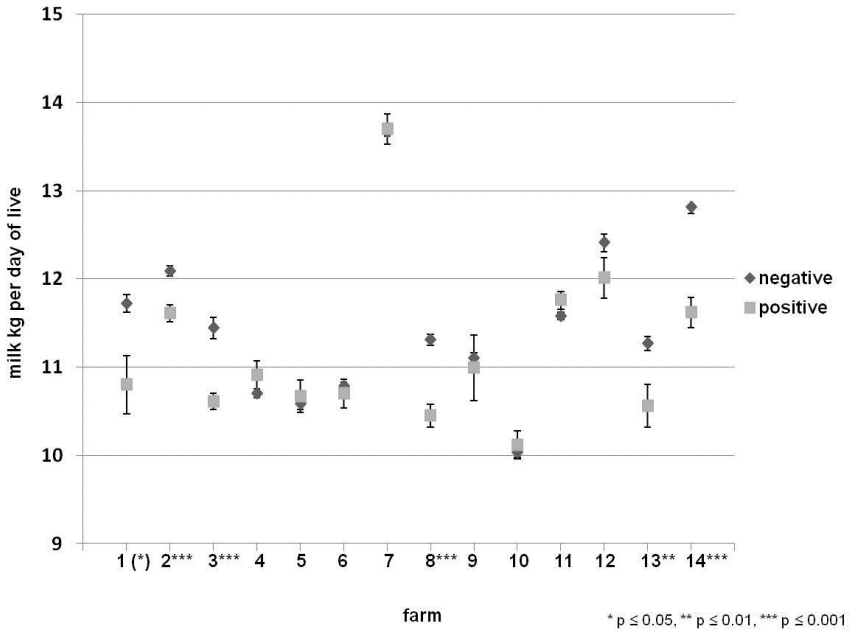

Figure 3: LS means for the average 305-day milk yield in kg for the interaction between farm and paratuberculosis status tested by fecal culture.

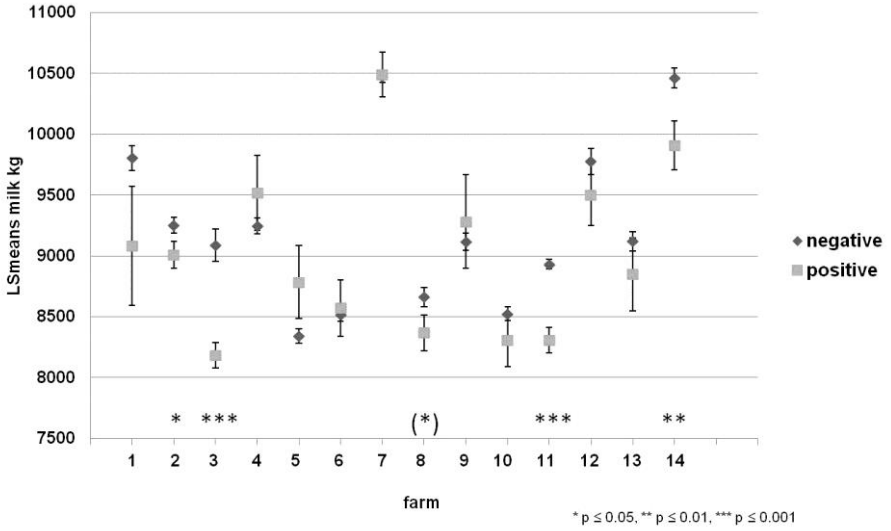

Figure 4: LS means for the milk per day of live in kg for the interaction between farm and paratuberculosis status tested by fecal culture.

DISCUSSION

The results of the present study confirmed the hypothesis that MAP positive animals show a decrease in the milk yield compared to negative animals. The reduction in milk production in MAP- positive animals is on average 225 kg (± 375 kg) and is equivalent to a milk reduction

of 2.4 %. This confirms the results reported by Hendrick *et al*. (2005) with losses in milk between 2 % and 6 %, depending on the diagnostic test used.

In general, the results about the decrease in milk kg in positive animals from the literature are heterogeneous and range between 206 kg and 1,364 kg per lactation (Shook *et al*., 2012, Raizman *et al*., 2009, Richardson and More, 2009, Sorge *et al*., 2011, Tiwari *et al*., 2007). One reason for this wide range of differences between MAP positive and negative tested animals could be the difference in the tests used or in the definition of MAP status itself. Some studies used a single test to classify the MAP status of animals while in other studies multiple test results for classification were combined to define positive tested animals.

A second major reason for the differing results is probably the highly significant interaction between farm and test results shown in our study (figure 3 and figure 4). In some farms positively tested animals do not show any decrease in milk production. From the results shown in figure 3 it can be seen that the level of milk production in the farms in our study does not have an influence on the difference between positive and negative tested cows. In both farms, 7 and 14, the average is higher than 10,000 kg of milk per lactation, but while in farm 7 no difference between positive and negative animals is observed, this difference is significant with 554 kg in farm 14. The same differing results are observed for farms 3, 6 and 10 with an average of milk kg per lactation of around 8,500 kg.

There is more a tendency that the difference between positive and negative tested cows in milk production traits is depending on the herd prevalence. We can demonstrate that a lower herd prevalence level leads to lower differences by comparing the herd prevalence of the four farms with the significant lower milk production for positive tested animals with the prevalence of the other farms. The farms with the significant differences show prevalence rates between 12.5 % and 64 %, while the others have prevalence rates below 12% and the farms with nearly equal milk production of positive and negative tested animals have the lowest prevalence rates. Beside this prevalence effect, the time of infection probably has an influence on milk production. The above mentioned four farms with the highest prevalence rates also have the highest percentage of cows tested positive already in the first lactation and in these farms in later lactations no higher prevalence rates are observed, as described by Küpper *et al*. (2012).

Gonda *et al*. (2007) also reported a decrease in fat kg (10-23 kg) and protein kg (8.3-18 kg). In the present study we could also observe a significant decrease in fat kg and in protein yield. The analysis of the fat- and protein- percentages offers no differences between positive and negative animals. So the significant reduction in fat- and protein- yield would be a result of the milk yield decrease, whereas the milk composition in total will be unchanged.

As measurement of reproductive efficiency often the calving interval or the days open are used. The results of different studies are conflicting. Raizman *et al*. (2009) found no significant difference between positive and negative cows. Johnson-Ifearulundu *et al*. (2000) reported a significant increase in days open in ELISA positive cows, but no significant differences in fecal tested cows. Lombard *et al*. (2005) reported that cows with strong positive ELISA test results showed fewer days open than cows with negative ELISA test results. The study of Ansari-Lari *et al*. (2012) showed nearly significance in calving interval between positive and negative tested herds. In our study we are in agreement with results of the latter study, observing a nearly significant influence ($p = 0.051$) of the MAP-test result on the calving interval. As shown already for milk production traits, the results for calving interval are probably also dependent on the test used and furthermore influenced by a significant interaction between farm and MAP status. Our results show that this interaction is significant also for first calving age ($p \leq 0.05$) and although not significant for calving interval, differences between 16 days of longer calving interval for positive tested animals to 31 days shorter calving interval for the positive animals are observed within farms.

When taking into account all lactations of tested cows an increasing effect with higher lactation numbers for kg of milk per day of life is observed. Within the first lactation there is no difference between positive and negative tested animals observed while in later lactations this difference is increasing and highly significant (figure 2). This effect could be a cumulative effect of the reduced milk production per lactation of MAP infected animals and the increased calving interval of the positive tested cows versus negative ones.

Other studies used as reference negative-tested in certified-free herds (Beaudeau *et al*., 2007) which would definitely have an influence on the results in case of significant interaction effects between farm and MAP status as shown in our study.

Because of a lack of knowledge regarding all costs connected with MAP (e.g. culling costs, costs regarding food intake of positive and negative animals) we could not account all costs of MAP infection. However, based on the fact that the average milk price in Germany varied between 31.56 cents and 34.77 cents per liter in the last years the annual losses of income are between 71 € (±118 €) up to 78 € (±130 €) per cow as result of the lack in milk yield.

CONCLUSION

The present results show that milk yield and milk parameters in dairy cows are affected by the MAP status of the animals and a high significant interaction between farm and MAP status exists. Results of the interaction between farm and MAP infection status show that these differences between positive and negative tested cows in milk production are probably dependent on herd prevalence but not on average milk yield of farm.

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REFERENCES

Ansari-Lari M, Haghkhah M, Mahmoodi F (2012) Association of *Mycobacterium avium* subspecies *paratuberculosis* infection with milk production and calving interval in Iranian Holsteins. Trop Anim Health Prod 44, 1111-1116

Benedictus G, Dijkhuizen A, Stelwagen J, (1987) Economic losses due to paratuberculosis in dairy cattle. Vet Rec 121, 142–146

Beaudeau F, Belliard M, Joly A, Seegers H (2007) Reduction in milk yield associated with *Mycobacterium avium* subspecies *paratuberculosis* (Map) infection in dairy cows. Vet Res 38, 625-634

Chi J, Van Leeuwen JA, Weersink A, Keefe GP (2002) Direct production losses and treatment costs from bovine viral diarrhea virus, bovine leucosis virus, *Mycobacterium avium* subspecies *paratuberculosis*, and Neospora caninum. Prev Vet Med 55, 137–153

Collins MT, Gardner IA, Garry FB, Roussel AJ, Wells SJ (2006) Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. JAVMA 229, 1912-1919

Donat K, Schau U, Soschinka A, Köhler H (2012) Herd prevalence studies of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in cattle using serological tests: opportunities, limitations and costs. Berl Münch Tierärztl Wochenschr 125, 361–370

Gonda MG, Kirkpatrick BW, Shook GE, Collins MT (2007) Identification of a QTL on BTA20 affecting susceptibility to *Mycobacterium avium* ssp. *paratuberculosis* infection in US Holsteins. Anim Genet 38, 389-396

Hendrick SH, Kelton DF, Leslie KE, Lissemore KD, Archambault M, Duffield TF (2005) Effect of paratuberculosis on culling, milk production, and milk quality in dairy herds. J Am Vet Med Assoc 227, 1302–1308

Johnson-Ifearulundu HJ, Kaneene JB, Sprecher DJ, Gardiner JC, Lloyd JW (2000) The effect of subclinical M*ycobacterium paratuberculosis* infection on days open in Michigan, USA, dairy cows. Prev Vet Med 46, 171-181

Küpper J, Brandt H, Donat K, Erhardt G (2012) Heritability estimates for *Mycobacterium avium* subspecies *paratuberculosis* status of German Holstein cows tested by fecal culture. J Dairy Sci 95, 2734-2739

Lombard JE, Garry FB, McCluskey BJ, Wagner BA (2005) Risk of removal and effects on milk production associated with paratuberculosis status in dairy cows. J Am Vet Med Assoc 227, 1975–1981

McKenna SLB, Keefe GP, Tiwari A, Van Leeuwen JA, Barkem HW (2006) Review Article: Johne's disease in Canada. Part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47, 1089–1099

Nielsen SS, Enevoldsen C, Gröhn YT (2002) The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. Prev Vet Med 54, 1-10

Nielsen SS, Toft N (2008) Ante mortem diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon-γ assay and faecal culture techniques. Vet Microbiol 129, 217-235

Nordlund KV, Goodger W, Pelletier J, Collins M (1996) Associations between subclinical paratuberculosis and milk production, milk components, and somatic cell counts in dairy herds. J Am Vet Med Assoc 208, 1872–1876

Ott SL, Wells SJ, Wagner BA (1999) Herd-level economic losses associated with Johne's disease on US dairy operations. Prev Vet Med 40, 179-192

Raizman EA, Fetrow J, Wells SJ, Godden SM, Oakes MJ, Vazquez G (2007) The association between *Mycobacterium avium* ssp. *paratuberculosis* fecal shedding or clinical Johne's disease and lactation performances on two Minnesota, USA dairy farms. Prev Vet Med 78, 179-195

Raizman EA, Fetrow J, Wells S (2009) Loss of income from cows shedding *Mycobacterium avium* subspecies *paratuberculosis* prior to calving compared with cows not shedding the organism on two Minnesota dairy farms. J Dairy Sci 92, 4929-4936

Richardson EKB, More SJ (2009) Direct and indirect effects of Johne's disease on farm and animal productivity in an Irish dairy herd. Ir Vet J 62, 526-532

Shook GE, Chaffer M, Wu X-L, Ezra E (2012) Genetic parameters for paratuberculosis infection and effects of infection on production traits in Israeli Holsteins. Anim Genet 43, 56-64

Sorge US, Kelton D, Lissemore K, Godkin A, Hendrick S, Wells S (2010) Attitudes of Canadian dairy farmers toward a voluntary Johne's disease control program. J Dairy Sci 93, 1491–1499

Sorge US, Lissemore K, Godkin A, Hendrick S, Wells S, Kelton D (2011) Association between paratuberculosis milk ELISA results, milk production, and breed in Canadian dairy cows. J Dairy Sci 94, 754-761

Sweeney RW, Collins MT, Koets AP, Mc Guirk SM, Roussel AJ (2012) Paratuberculosis (Johne's disease) in cattle and other susceptible species. J Vet Intern Med 26,1239-50

Tiwari A, Van Leeuwen JA, McKenna SLB, Keefe GP, Barkem HW (2006) Review Article: Johne's disease in Canada. Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J 47, 874–882

Tiwari A, Van Leeuwen JA, Dohoo IR, Keefe GP, Haddad JP, Tremblay R, Scott HM, Whitings T (2007) Production effects of pathogens causing bovine leukosis, bovine viral diarrhea, paratuberculosis, and neosporosis. J Dairy Sci 90, 659–669

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

Windsor PA, Whittington RJ (2009) Evidence for age susceptibility of cattle to Johne's disease. Vet J doi: 10.1016/j.tvjl.2009.01.007

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4. SHORT COMMUNICATION :GENETIC ASSOCIATION BETWEEN *NOD2* POLYMORPHISM AND INFECTION STATUS BY *MYCOBACTERIUM AVIUM* SSP. *PARATUBERCULOSIS* IN GERMAN HOLSTEIN CATTLE

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Summary

The aim of the study was the analysis of the *nucleotide-binding oligomerization domain containing 2* (*NOD2*, formerly *CARD15*) as a candidate gene for *Mycobacterium avium* ssp. p*aratuberculosis* infection in cattle. Eleven SNPs in the *NOD2* gene were identified, and finally, four SNPs were included in a case–control study using 324 German Holstein cows tested for paratuberculosis using fecal culture and ELISA. The SNP (GenBank) AY518738S04:g.521G>A in exon 4 showed a significant association between the fecal culture status of the animals and *NOD2* allele variants. The other three SNPs showed no associations in German Holstein cows.

Keywords: bovine, candidate gene, *CARD15*, fecal culture, paratuberculosis, *nucleotidebinding oligomerization domain containing 2*

The causative agent *Mycobacterium avium* ssp. *paratuberculosis* (MAP) is responsible for the chronic intestinal disease paratuberculosis (ParaTB) or Johne's disease in ruminants. The relationship between MAP and Crohn's disease in humans has been discussed (Singh & Gopinath 2011), but the causal role of MAP has not been confirmed thus far (Mendoza *et al*. 2009). In humans, more than 30 genes with an influence on Crohn's disease have been described (Van Limbergen *et al*. 2009). Also, a few studies in cattle with potential genetic loci associated with MAP have been published (Purdie *et al*. 2011). One of these genes of interest is the *nucleotide-binding oligomerization domain containing 2* (*NOD2*) gene, encoding an intracellular receptor, formerly known as *caspase recruitment domain, member 15* (*CARD15*) (Pinedo *et al.* 2009; Ruiz-Larrañaga *et al.* 2010). The NOD2 protein recognizes components of the mycobacterial cell wall (Oh *et al.* 2005) and induces activation of the NF-κB proinflammatory signaling pathway (Ogura *et al.* 2001). In humans, polymorphisms in the *NOD2* gene are associated with an increased risk of Crohn's disease (Hugot *et al.* 2001; Ogura *et al.* 2001). Two polymorphisms in *NOD2* were described to be associated with the susceptibility to MAP infection, one (*AH013658:c.1908C>T*) in Spanish Holstein Friesian cattle by Ruiz-Larrañaga *et al.* (2010) and one (*AY518737:E4+921*) in German Holstein cattle

by Hinger (2009). Another association (*AH013658:c.2197T>C*) was described in Brahman–Angus (*B. taurus x B. indicus*) crosses by Pinedo *et al.* (2009). The aforementioned results of association studies including *NOD2* and MAP susceptibility are inconsistent. A possible reason could be the different breeds and crosses that were analyzed as well as the different phenotypic characterizations of MAP infection that were used.

Therefore, in the first step of this study, 28 German Holstein cows were analyzed to detect genetic variability in *NOD2* on BTA18. All 12 exons, the 5' and 3' untranslated region (UTR), and flanking parts of the introns were amplified by PCR (Table S1). Resulting PCR products were sequenced in both directions with one of the PCR primers.

In total, we found 11 SNPs in the *NOD2* gene (Table S2): one in exon 4, one in exon 12, three in the 3' UTR, one in the 3' UTR flanking region and five in intron regions (intron 1, 4, 8 and 10). Four of the SNPs (intron 10, exon 12 and two in the 3' UTR) were described before by Taylor *et al.* (2006) and Ruiz-Larrañaga *et al.* (2010), and one SNP (exon 4) was described by Hinger (2009).

In the second step, we searched in a data set of 11 285 German Holsteins, tested in 2008 and 2009 for MAP by fecal culture (AVID 2007) for positive animals. To exclude stratification effects, every positive animal was matched to one fecal negative cow as control using the following criteria: same farm, same age (or older) and same sire. In total, we got 162 fecal positive and 162 matching fecal negative animals for the case–control study. Additionally, all animals were tested serologically by four different commercial ELISA tests. To genotype SNP No. 1 (*AY518738S04:g.521G>A*) in exon 4 and the two SNPs in the 3' UTR (SNP No. 3: *AY518738S11:g.2083* and SNP No.4: *JX448702:g.2580*), two PCR-restriction fragment length polymorphisms were developed. One additional SNP in the 3' UTR (SNP No. 2: *AY518738S11:g.1633C>A*) was analyzed by amplification refractory mutation system PCR (Newton *et al.* 1989).

The differences between the allele frequencies of the polymorphism in the *NOD2* gene were analyzed by chisquare test and Fisher's exact test using SAS system software (SAS 9.1, SAS Institute Inc.), using fecal positive or negative as the phenotype definition. The haplotype frequencies, linkage disequilibrium block and a possible association with the haplotypes and

fecal MAP status were estimated with HAPLOVIEW (Barrett *et al*. 2005). These four SNPs were all in Hardy–Weinberg equilibrium.

For a second analysis, we considered a case definition of animals positive for any of the four ELISA tests vs. controls that were negative for all four ELISA tests. For this analysis, we used a subset of all animals with 99 cases and 99 controls that matched the previous criteria.

The results of the estimated allele frequencies are shown in Table 1. Statistical analysis estimated significant differences (*P = 0.0056*) in allelic frequencies between fecal culture positive and negative animals for SNP No. 1. This indicates an association between infection status by fecal culture and allele variants. In contrast to the results of Hinger (2009), this SNP showed no association with the infection status tested by ELISA (Table 1). An explanation for varying results could be the differences in allele frequencies between the two studies. Nevertheless, associations detected with low allele frequency should be interpreted with caution. It is still not clear whether NOD2 is more involved in shedding (measured by fecal culture) or in the immune stimulation (measured by ELISA).

No associations between the fecal culture MAP status and the SNPs No. 2, 3 and 4 could be demonstrated, even if we analyzed their association in the ELISA subset.

Ruiz-Larrañaga *et al.* (2010) analyzed the association between MAP infection and known SNPs of the *NOD2* gene described by Taylor *et al*. (2006). They found four polymorphic SNPs in Spanish Holstein Friesian. In the present work using German Holstein, the first of these SNPs was monomorphic (Table S2). In the Ruiz-Larrañaga *et al.* (2010) study, only the SNP *AH013658:c.1908C*, identical to SNP No. 3 in the present work, was more frequent in infected Spanish Holstein Friesians than in healthy ones. However, this relationship could not be confirmed in Dutch Holstein Friesians (Ruiz-Larrañaga *et al.* 2010), which is in accordance with the results of the present study. Additionally, we could confirm the findings of Ruiz-Larrañaga *et al.* (2010) that there is no association between the 3' UTR region SNP *AH013658:c.*1458* (identical to SNP No. 2 in the present study) and the risk of MAP infection.

Table 1: Allelic frequencies (%) and significance of association of the SNPs g.521G>A (No. 1), g.1633C>A (No. 2), g.2083C>T (No. 3) and g.2580G>T (No. 4) in the case control study. All German Holstein cows tested for MAP with fecal culture (fecal culture positive n=162 or negative n=162) and with four different ELISA tests each (ELISA: positive to any of the four ELISA tests n=99 vs. negative to all four ELISA tests n=99).

No.	SNP	Allele	Fecal culture (N=324)			ELISA (N=198)			Other studies				
			Positive	Negative	Association	Positive	Negative	Association	Positive	Negative	Association	Diagnostic test	References
1	g.521	G	96.41	90.97		95.26	93.30		94.36	96.22			
					**			n.s.			**	ELISA	Hinger 2009
		A	3.59	9.03		4.74	6.70		5.64	3.78			
2	g.1633	C	57.23	57.59		56.63	54.55		58.7	62.4		fecal culture or PCR or ELISA	Ruiz-Larrañaga *et al.* 2010
					n.s.			n.s.			n.s.		
		A	42.77	42.41		43.37	45.45		41.3	37.6			
3	g.2083	C	85.17	85.85		85.71	87.37		88.7	79.3		fecal culture or PCR or ELISA	Ruiz-Larrañaga *et al.* 2010
					n.s.			n.s.			*		
		T	14.83	14.15		14.29	12.63		11.3	20.7			
4	g.2580	G	95.91	92.77		95.41	94.44						
					n.s.			n.s.					
		T	4.09	7.23		4.59	5.56						

**p≤0.01
*p≤0.05
n.s., Not significant.

In our study, the SNP at position c.2197, mentioned by Pinedo *et al.* (2009) in MAP infected Brahman–Angus cattle, is monomorphic, which is in agreement with Taylor *et al.* (2006). Consequently, we could not find any association between this SNP and the MAP infection status in cows.

Some of the SNPs show high linkage disequilibrium as shown in Fig. 1. Using the confidence interval (Gabriel *et al.* 2002), no highly frequent haplotype block could be defined, and no association with the MAP status could be found.

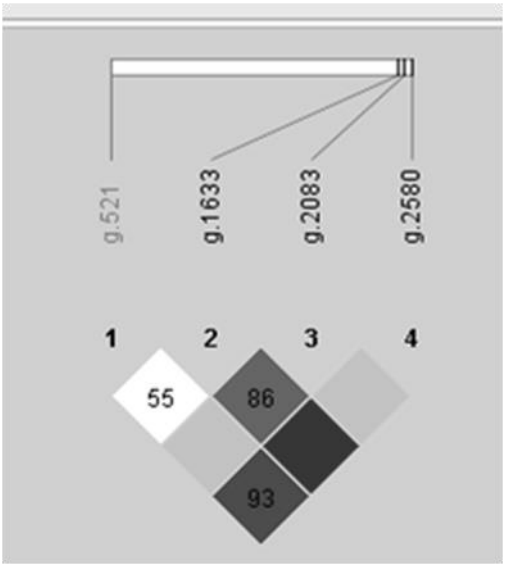

Figure 1: Linkage disequilibrium and haplotype structure of the *NOD2* gene. Haplotype block structure was determined using the HAPLOVIEW program (Barrett *et al.* 2005). Each box represents the D' values corresponding to each pairwise SNP. Most frequent haplotypes (in order, SNP-No.: 1, 2, 3 and 4): GACG with a frequency of 41.0%, GCCG with a frequency of 37.9% and GCTA with a frequency of 13.3%.

In conclusion, we found an association between the SNP *AY518738S05:g.521G>A* in exon 4 and the MAP infection status detected by fecal culture. Nevertheless, we could not confirm the role of the *NOD2* gene related to MAP susceptibility in ELISA test results. The differences in allelic frequencies of the cases and controls compared with other studies could be due to the lack of standardized diagnostic tests for MAP infection. But as in the other studies, we

could demonstrate that the candidate gene *NOD2* is likely associated with the MAP status, although the causative SNP or haplotype is not identified so far.

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References

AVID. (2007) Kultureller Nachweis von *Mycobacterium avium* subsp. *paratuberculosis* in Kot- oder Organproben. Deutsche Veterinärmedizinische Gesellschaft e. V., Gießen.

Barrett J.C., Fry B., Maller J. & Daly M.J. (2005) HAPLOVIEW: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263–5.

Gabriel S.B., Schaffner S.F., Nguyen H. *et al*. (2002) The structure of haplotype blocks in the human genome. *Science* **296**, 2225–9.

Hinger M. (2009) Untersuchungen genetischer Ursachen der unterschiedlichen Empfänglichkeit für die Paratuberkulose des Rindes anhand von Mikrosatelliten- und Kandidatengenanalyse. Doctoral thesis, Justus-Liebig-University Gießen. VVB Laufersweiler Verlag.

Hugot J.-P., Chamaillard M., Zouali H. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411**, 599–603.

Mendoza J.L., Lana R. & Diaz-Rubio M. (2009) *Mycobacterium avium* subspecies *paratuberculosis* and its relationship to Crohn's disease. *World Journal of Gastroenterology* **15**, 417–22.

Newton C.R., Graham A., Heptinstall L.E., Powell S.J., Summers C., Kalsheker N., Smith J.C. & Markham A.F. (1989) Analysis of any point mutation in DNA The amplification refractory system (ARMS). *Nucleic Acids Research* **17**, 2503–16.

Ogura Y., Bonen D.K., Inohara N. *et al*. (2001) A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease. *Nature* **411**, 603–6.

Oh H.-M., Lee H.-J., Seo G.-E. *et al.* (2005) Induction and localization of *NOD2* protein in human endothelial cells. *Cellular Immunology* **237**, 37–44.

Pinedo P.J., Buergelt C.D., Donovan G.A., Melendez P., Morel L., Wu R., Langaee T.Y. & Rae D.O. (2009) Association between CARD15/NOD2 gene polymorphisms and paratuberculosis infection in cattle. *Veterinarian Microbiology* **134**, 346–52.

Purdie A.C., Plain K.M., Begg D.J., de Silva K. & Whittington R.J. (2011) Candidate gene and genome-wide association studies of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and sheep: a review. *Comparative Immunology, Microbiology & Infectious Diseases* **34**, 197–208.

Ruiz-Larrañaga O., Carrido J.M., Iriondo M., Manzano C., Molina E., Koets A.P., Rutten V.P., Juste R.A. & Estonba A. (2010) Genetic association between bovine NOD2 polymorphisms and infection by *Mycobacterium avium* subsp. *paratuberculosis* in Holstein- Friesian cattle. *Animal Genetics* **41**, 652–5.

Singh S. & Gopinath K. (2011) Mycobacterium avium subspecies paratuberculosis and Crohn's regional ileitis: how strong is association? *Journal of Laboratory Physicians* **3**, 69–74.

Taylor K.H., Taylor J.F., White S.N. & Womack J.E. (2006) Identification of genetic variation and putative regulatory regions in bovine CARD15. *Mammalian Genome* **17**, 892–901.

Van Limbergen J., Wilson D.C. & Satsangi J. (2009) The genetics of Crohn's disease. *Annual Review of Genomics and Human Genetics* **10**, 89–116.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Information about the amplified *NOD2* regions, primer sequences, fragment sizes and annealing temperatures of PCR amplifications.

Table S2. SNPs identified in this study in the bovine *NOD2* gene.

Supplementary table 1: Information about the amplified *NOD2* regions, primer sequences, fragment sizes, and annealing temperatures of PCR amplifications. GeneBank sequences used for primer design were AC_000175.1 (selected region from 19 181 972 to 19 213 217) and AY518737.1

Primer sequences (5' --> 3', forward and reverse primer, respectively)	Amplified *NOD2* regions (exons in Roman numerals, introns in Arabic numerals)	Fragment size (bp)	Annealing temperature
gttttgtagacagacgctggagt atttcacaatctgggaggagaat	5'UTR (I – 1)	1 424	62°C
cttggtgtctgagggtacagc aggaggactatgacccacatctc	1[1] – II – 2[1]	690	62°C
tttattgtggtggaagagagagg ttacccttaaaatgagacgctga	2[1] – III – 3[1]	387	62°C
catgcatcacatctcatttcct[2] gcttcctcaggtacagttcgat[2]	3[1] – IV[1]	827	64°C
actcagcctcaagggcttct aggaacaatttgggcagcac	IV[1]	624	64°C
cctggaatttttgcatatcactt ttcgagaatagcttgagggtgct	IV[1] – 4[1]	804	53°C
gtagagactctgggtccccaat ccctgttcagaatgagacactg	4[1] – V – 5 – VI – 6[1]	753	56°C
ctttgctggtggaaaatgaagt tgagcttcctgtttattgagca	6[1] – VII – 7[1]	517	53°C
gaattggcattgagaatgagttc agtgtatctgattcagggcttcc	7[1] – VIII – 8[1]	852	64°C
gaagccttggatcagctacact attataagccatggaggaagca	8[1] – IX – 9[1]	561	56°C
gacccttacactttgcttgacc caccagcttgatgacacacttt	9[1] - X – 10[1]	458	56°C
attgggaatctcagacaggaaa acagttggtccctgcttaaaaa	10[1]– XI – 11[1]	417	62°C
ttacaaagcagcatcttcctga acaagggaaagatcaaaggtga	11[1] – XII - 3'UTR[1]	662	62°C
tcgggaagtaactgtttgcttt aacacttgcacagtacctgaca	3'UTR[1]	659	62°C
gtttacagggcaaaatcaaagg ttgcgtctctctcatctgtgtt	3'UTR[1]	519	62°C
attctccttgaagggaggagac gtcctaatgtatagcacaaggaact	3'UTR[1]	624	62°C
tggttgatttacagcgttgtgt[3] ttttccagcttatttgttttcc[3]	3'UTR[1]	777	56°C
ctccttgaagggaggagaca tggagacacttggagagaatagagt tggagacacttggagagaatagagg	3'UTR	232	62°C

[1]partly

[2]PCR conditions for PCR-RFLP with *Tsp45*I

[3]PCR conditions for PCR-RFLP with *Mse*I

Supplementary table 2: SNPs identified in this study in the bovine *NOD2* gene. SNP no. 1 – 4 were used for association studies

	Present study:			Confirmed by other authors:		
No.	SNP	Location	GenBank	SNP	GenBank	Reference
	g.1089A>G	Intron 1	JX448700			
1	g.521G>A	Exon 4	AY518738S04	E4+921	AY518737	Hinger 2008
	g.146T>G	Intron 4	AY518738S05			
	--	--	--	c.2634-459G>A (ss159816034)	AH013658	Ruiz-Larrañaga *et al.* 2010
	g.589T>C	Intron 8	JX448701			
	g.632G>A	Intron 10	AY518738S09			
	g.138A>G	Intron 10	AY518738S10	(rs43710287)	AH013658	Taylor *et al.*2006
	g.153A>T	Exon12	AY518738S11	c.3020A>T (rs43710288)	AH013658	Ruiz-Larrañaga *et al.* 2010; Taylor *et al.* 2006
	g.806G>A	3'-UTR	AY518738S11			
2	g.1633C>A	3'-UTR	AY518738S11	c.*1458C>A (rs43710289)	AH013658	Ruiz-Larrañaga *et al.* 2010; Taylor *et al.* 2006
3	g.2083C>T	3'-UTR	AY518738S11	c.*1908C>T (rs43710290)	AH013658	Ruiz-Larrañaga *et al.* 2010; Taylor *et al.* 2006
4	g.2580G>T	3'-UTR flanking region	JX448702			

5. PHENOTYPE DEFINITION IS A MAIN POINT IN GENOME-WIDE ASSOCIATION STUDIES FOR BOVINE MYCOBACTERIUM AVIUM SSP. PARATUBERCULOSIS

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Abstract

Paratuberculosis caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP) causes economic losses and is present in dairy herds worldwide. Different studies used different diagnostic tests to detect infection status and are the basis of genome-wide association (GWA) studies with inconsistent results. Therefore, the aim of this study was to identify and compare genomic regions associated with MAP susceptibility in the same cohort of cattle using different diagnostic tests. The GWA study was performed in German Holsteins within a case-control assay using 305 cows tested for MAP by fecal culture and additional with four different commercial ELISA-tests. Genotyping was performed with the Illumina Bovine SNP50 BeadChip. The results using fecal culture or ELISA test led to the identification of different genetic loci. Two single-nucleotide polymorphisms showed significant association with the ELISA-status. However, no significant association for MAP infection could be confirmed. Our results show that the definition of the MAP-phenotype has an important impact on the outcome of GWA studies for paratuberculosis.

Key Words: cattle, paratuberculosis, genome wide association, fecal culture, ELISA

Implications

Paratuberculosis, caused by the agent *Mycobacterium avium* ssp. *paratuberculosis* (MAP), occurs worldwide and leads to high economic losses, due to reduced milk production, reduced fertility and higher management costs in dairy farms. Heritabilities for the susceptibility to MAP infection range between 0.031 and 0.283, whereas fecal culture shows a higher genetic background than ELISA test results. Due to different test systems used for phenotypic characterization of the MAP status of the cattle, so far association studies lead to inconsistent results. Therefore it was the aim of this study to perform a case-control genome-wide association study with MAP fecal culture phenotyped German Holstein cows which were simultaneous tested with four different ELISA tests to contribute to the understanding of genetic variability involved in MAP susceptibility.

Introduction

Paratuberculosis or Johne's disease is a chronic intestinal disease especially in ruminants caused by the agent *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Infection with MAP occurs worldwide and leads to economic losses due to lower milk production, reduced fertility and costs associated with the management of infected animals (McKenna *et al.*, 2006). Estimated prevalence rates for MAP are among European cattle up to 20%, but at least 3 to 5% in some countries (Nielsen and Toft, 2009). Due to a lack of country wide testing schemes, the long incubation time, subclinical phases (Chiodini *et al.*, 1984) and problems with test method accuracy (Kohl *et al*., 2012), it is difficult to make a statement about prevalence rates. In 2007, the U.S. Departement of Agriculture's (USDA) National Animal Health Monitoring System reported, that 68% of dairy herds in the United States were found to be infected with MAP, whereas 21.6% of these farms had at least 10% of cows infected with MAP. In contrast herd prevalence rates in Europe range from 6.9% in Austria (Dreier *et al.*, 2006) to 54.7% in the Netherlands (Muskens *et al*., 2000). Another aspect is the controversially discussed relationship between MAP and Crohn's disease in humans whereas the causal role of MAP has not been confirmed so far (Mendoza *et al*., 2009).

As an infection with MAP is not treatable it is important to control the infection with MAP in farm animals, by (a) preventing transmission of the organism to susceptible animals by management strategies, (b) by identifying and culling infected animals, and/or by clarifying the genetic background of MAP susceptibility to improve selection options. Several studies have estimated heritabilities for paratuberculosis susceptibility. Depending on the test method for MAP and the model used, heritability for MAP infection status varies between 0.031 and 0.283 (Hinger *et al*., 2008; Küpper *et al*., 2012; van Hulzen *et al*., 2012). Thereby the fecal culture test results shows a higher genetic variation than ELISA test results. Fecal culture tests are also often used as standard when calculating sensitivity and specificity for ELISA tests (Donat *et al*., 2012).

Recently the potential genetic loci associated with MAP in cattle have been reviewed (Purdie *et al*., 2011). These loci were identified by testing candidate genes, by genome-wide linkage or association studies. A genome wide linkage analysis identified a quantitative trait locus associated with MAP susceptibility on *Bos taurus* chromosome 20 (BTA 20) (Gonda *et al*.,

2007). Polymorphisms with significant associations to MAP susceptibility were found in the following candidate genes: *CARD15* (Pinedo *et al*., 2009a; Ruiz-Larranaga *et al*., 2010a), *IL10* (Verschoor *et al*., 2010), *TLR1*, *TLR2*, *TLR4* (Mucha *et al*., 2009; Purdie *et al*., 2011) and *SLC11A1* (Pinedo *et al*., 2009b; Ruiz-Larranaga *et al*., 2010b).

Table 1 *Comparison of genome wide association studies related to MAP-infection in dairy cows*

Author		Definition case	Definition control	n
Settles *et al*., 2009	Grouping 1	Tissue culture pos (regardless of fecal culture)	tissue culture neg (regardless fecal culture)	Pos. = 90 Neg.=119
	Grouping 2	Fecal culture pos (regardless of tissue culture)	Fecal culture neg (regardless of tissue culture)	Pos. = 41 Neg.=168
	Grouping 3	Tissue culture pos and fecal culture neg	Tissue culture and fecal culture neg	Pos. = 54 Neg.=112
	Grouping 4	Tissue culture pos and fecal culture pos	Tissue culture and fecal culture neg	Pos. = 25 Neg.= 112
Minozzi *et al*., 2010	Sample set 1	ELISA pos	ELISA neg	Pos. = 483 Neg.= 783
	Sample set 2	ELISA pos	ELISA neg	Pos. = 140 Neg.= 137
Pant *et al*., 2010		ELISA pos	ELISA neg and >5.8 years	Pos. = 90 Neg.= 142
Kirkpatrick *et al*., 2011	Sample set 1	ELISA pos or fecal pos	Specific reference population	Pos. = 248 Neg.= 1 546
	Sample set 2	ELISA pos	Specific reference population	Pos. = 307 Neg.= 1 546
Minozzi *et al*., 2012	Grouping 1	ELISA pos or tissue culture pos	ELISA neg or tissue culture neg	Pos. = 590 Neg.= 600
	Grouping 2	ELISA pos or tissue culture pos	tissue culture neg	Pos. = 590 Neg.= 117
van Hulzen *et al*., 2012		Deregressed proofs for sires (n = 192) calculated from log-transformed ELISA test results in milk of their daughters (n = 265 290)		

MAP = *Mycobacterium avium* ssp. *paratuberculosis.*

The first genome-wide association (GWA) study using the Illumina Bovine SNP50 BeadChip related to MAP susceptibility was published by Settles *et al*. (2009). In this study, regions on

BTA 3 and 9 were identified with a high significance to MAP status tested by MAP culture in feces and tissue in US Holsteins. In different GWA studies several associations were found on different chromosomes (Minozzi *et al*., 2010; Pant *et al*., 2010; Kirkpatrick *et al*., 2011; Minozzi *et al*., 2012; van Hulzen *et al*., 2012), summarized in supplementary Table S1. These studies provide in general evidence for the existence of genomic regions associated with MAP infection in dairy cows. Nevertheless the results are not consistent, probably because different test methods and study designs were used as summarized in Table 1, which may directly influence the statistical power.

Therefore, it was the aim of this study to perform a case-control GWA study with MAP fecal culture tested German Holstein cows which were simultaneous tested with four different ELISA tests, for a better understanding of associated genetic loci found by different paratuberculosis tests.

Materials and methods

Dataset and phenotyping

In 2008 and 2009 11 285 German Holstein herdbook cows were tested for MAP infection by fecal culture, within a voluntary program in Thuringia, Germany (Donat *et al*., 2012). For the case-control study 152 fecal culture positive, animals were chosen as cases and following their pedigree information, we assigned every positive animal one animal as control. To exclude possible stratification factors we used the following criteria for the 153 fecal negative animals of the control group: (1) same farm, (2) same sire and (3) same age (± three month). From these 305 animals blood samples were collected via K-EDTA Monovette® (Sarstedt, Nümbrecht). The samples were centrifuged (840xg) for 20 min. Buffy coat and plasma from each sample were kept at -20°C until further usage.

In addition to fecal culture, we tested in plasma of all the 305 animals included in the case-control study the MAP antibody titer with four different commercial blood ELISA tests: CATTLETYPE® MAP Ab (Labor Diagnostik Leipzig, Germany), ID Screen® (ID.vet Montpellier, France), Parachek® (Prionics Schlieren-Zürich, Switzerland), and Pourquier® Mycobactin

(IDEXX Laboratories Inc. Westbrook, USA). These four tests were all accredited for Germany in 2010. Test executions, as well as the cut-offs for the qualitative analysis were done according to manufactures information.

Additional to the classification as fecal case and control, we regrouped the 305 animals according to their ELISA test results. On the one hand positive is defined whenever one animal was positive at least in one of the four different ELISA test results ($ELISA^{+}$), and on the other hand positive is defined whenever one animal was positive in all four different ELISA test results ($ELISA^{4+}$). In this analysis we considered only the ELISA tests to analyze the differences between ELISA test methods.

Illumina SNP50 BeadChip

DNA was extracted from the buffy coat according to Montgomery and Sise (1990) and genotyped using either the Illumina SNP50 BeadChip v1 with a total of 54 001 single-nucleotide polymorphisms (SNPs) for 178 animals (mean spacing between the SNPs of 51.5 kb) or the second generation Illumina SNP50 BeadChip v2 containing 54 609 SNPs for 127 animals (mean spacing between the SNPs of 49.4 kb). In total 48 186 SNPs are available in both BeadChips and are used for the statistical analysis. Samples were genotyped within the genotyping platform of the German Holstein breeding association routinely used for SNP typing for estimating genomic breeding values.

Statistical analysis and quality control

Quality control parameters for the SNP-data were measured with R using the "check.marker" function by GenABEL package (Aulchenko *et al*., 2007). Markers not displaying Hardy Weinberg equilibrium ($P \leq 5\%$), with low call (< 95%) rates or minor allele frequencies (maf < 2%) were excluded as well as markers with more than 5% of missing data. Also animals with low call rates (< 95%) and high identical by state (IBS ≥ 95) were excluded. The "qtscore" function for a binary trait of the GenABEL package (Aulchenko *et al*., 2007) was used for the GWA study. The data were not pruned. Uncorrected *P*-values between

$5x10^{-7}$ and $5x10^{-5}$ were considered as moderately significant and *P*-values $<5x10^{-7}$ provided strong evidence of association SNPs without known map position were excluded for the Manhattan plots.

Results

The results of the ELISA test in relation to the fecal test results are shown in Tables 2 and 3. From the total 305 animals (152 fecal positive and 153 fecal negative), 84 animals were identified as positive with all test methods and 126 animals were tested negative with all test methods. A smaller number of animals are detected as positive by ELISA test. From the 152 fecal positive tested animals 58 and 68, respectively, could not be detected by $ELISA^{+}$ or $ELISA^{4+}$ as positive.

Table 2 *Comparison of fecal culture and $ELISA^{+}$ test results for MAP infection*

		Fecal positive	Fecal negative	Total
$ELISA^{+}$	Positive	94	27	121
	Negative	58	126	184
	Total	152	153	305

MAP = *Mycobacterium avium* ssp. *paratuberculosis.*
ELISA positive is defined whenever animals were positive in one of the four different ELISA test results.

Table 3 *Comparison of fecal culture and $ELISA^{4+}$ test results for MAP infection*

		Fecal positive	Fecal negative	Total
$ELISA^{4+}$	Positive	84	17	101
	Negative	68	136	204
	Total	152	153	305

MAP = *Mycobacterium avium* ssp. *paratuberculosis.*
ELISA positive is defined whenever animals were positive in four of the four different ELISA test results.

GWA analysis

Quality control. A total of 6414 markers were excluded from the study due to low minor allele frequency and 1896 were excluded due to low call rate. In total, 40 079 markers and 305 animals were included in the statistical analysis.

Fecal positive v. fecal negative. For the genome wide association analysis 152 fecal culture positive and 153 fecal culture negative animals were used. Figure 1 shows the results of the GWA scan. Table 4 summarizes the SNPs that show strongest association with MAP status based on serological testing either in one ($ELISA^{+}$) or in four ($ELISA^{4+}$) different ELISA tests. No significant association between the SNPs and the fecal test results could be demonstrated.

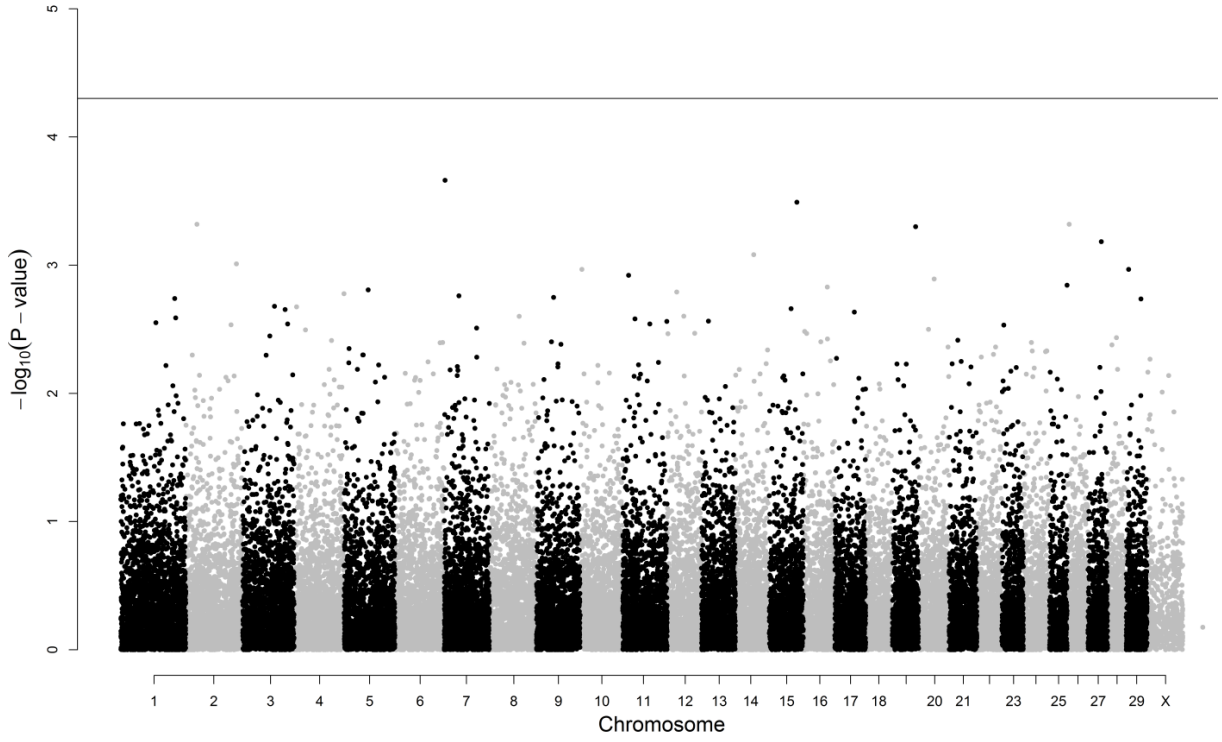

Figure 1 Association of single-nucleotide polymorphisms (SNPs) with *Mycobacterium avium* ssp. *paratuberculosis* (MAP)-infection in Holsteins phenotyped by fecal culture. Manhattan plot of $-\log_{10}$ *(P*-values) with results of the whole-genome scan for an association of loci with *Mycobacterium avium* ssp. *paratuberculosis* fecal culture positive tested German Holstein cows. The straight line at $-\log_{10}(5 \times 10^{-5})$ indicates a moderate significance level.

Table 4 *List with P-values of the most associated SNPs with MAP fecal culture positive and with MAP ELISA positive tested Holsteins*

SNP	BTB-01407725	ARS-BFGL-NGS-64801	BTB-01629524	BTB-01629562	UA-IFASA-7571	BTB-01513424	BTA-89505-no-rs	ARS-BFGL-NGS-57708
Chromosome	7	15	2	26	19	27	14	2
Position	2137411	68665371	27967801	5237427	58038747	33269102	45717997	125894202
N	305	304	305	305	300	305	305	305
Minor/major allele	G/A	G/A	G/A	G/A	A/G	A/G	A/C	G/A
Allele frequency	0.08	0.03	0.43	0.43	0.12	0.03	0.07	0.43
Call rate	1	0.996721	1	1	0.983607	1	1	1
FECAL p=	2.18e-04	3.23e-04	4.79e-04	4.79e-04	5.01e-04	6.55e-04	8.29e-04	9.78e-04
Odd ratio	0.29	25.18	0.55	0.55	2.53	7.65	0.30	0.57
$ELISA^{+}$ p=	0.306	0.402	5.58e-02	5.58e-02	0.176	9.71e-02	0.509	8.95e-02
Odd ratio	0.72	1.55	0.71	0.71	1.41	2.14	0.80	0.74
$ELISA^{4+}$ p=	0.193	0.154	4.86e-03	4.85e-03	6.24e-02	0.15416	0.283	1.03e-02
Odd ratio	0.63	2.09	0.56	0.56	1.61	1.92	0.67	0.60

SNP = single-nucleotide polymorphism; MAP = *Mycobacterium avium* ssp. *paratuberculosis.*
ELISA positive is defined whenever animals were positive in one (ELISA+) or respectively four (ELISA4+) of the four different ELISA test results.

ELISA positive vs. ELISA negative. In the first analysis ($ELISA^{+}$, where positive is defined whenever one animal was positive in one of the four different ELISA test results) 121 animals were used as case and 184 animals for control. The GWA scan (Figure 2) of this dataset shows no strong associations with *P*-values of $<5x10^{-7}$. Two SNPs with moderate associations were found: one on BTA 5 (position: 59 694 618, $P = 1.43x10^{-5}$) and the other on BTA 25 (position: 23 151 542, $P = 1.89x10^{-5}$). In the second analysis ($ELISA^{4+}$), where cases (n=101) are defined as animals with positive test results in all four ELISA tests, only the SNP on chromosome 5 (position: 59 694 618, $P = 1.93x10^{-5}$) shows a moderate association to MAP infection (Figure 3). The results are summarized in Table 5.

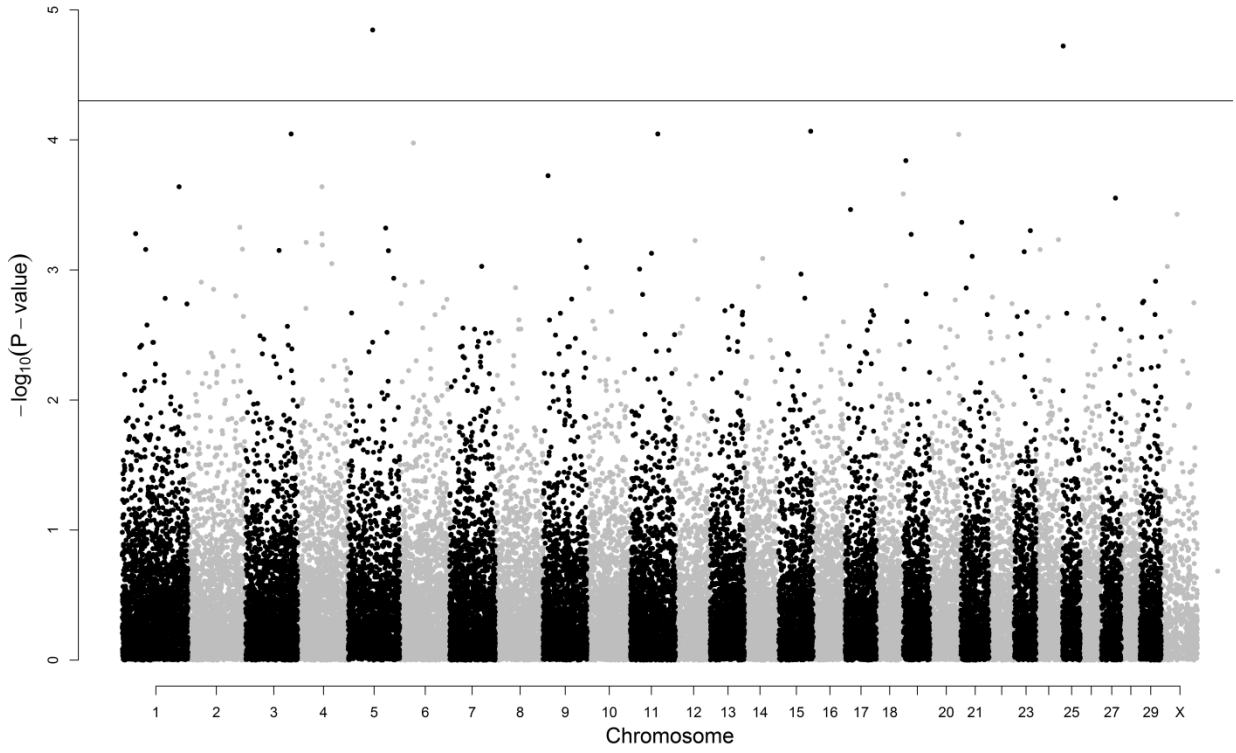

Figure 2 Association of single-nucleotide polymorphisms (SNPs) with *Mycobacterium avium* ssp. *paratuberculosis* (MAP)-infection in Holsteins phenotyped by ELISA^{+}. Manhattan plot of $-\log_{10}$ *(P*-values) with results of the whole-genome scan for an association of loci with *Mycobacterium avium* ssp. *paratuberculosis* ELISA^{+} positive tested German Holstein cows. Positive is defined whenever one animal was positive in at least one of four different ELISA test results (ELISA^{+}), regardless of fecal culture results The straight line at $-\log_{10}$ (5×10^{-5}) indicates a moderate significance level.

Discussion

The differences in the number of positive/negative animals between fecal test results and ELISA test results (Tables 2 and 3) within the same population shows the diagnostic complexity and, therefore, the difficulty to define the phenotype for a case-control study in a correct way. Also the differences between the four ELISA test results reflect the same problem. The differences between the fecal test results and ELISA test results are caused by the specific pathogenesis of paratuberculosis (Collins, 2003). The synthesis of antibodies is causally linked to the stage of the disease. First, after MAP infection the cell-mediated immune response to MAP is an early event in the pathogenesis, whereas the humoral immune response follows in later stages of the disease. Because of the different stages in

the pathogenesis of paratuberculosis, it is evident to get different test results within the same animal, depending on time of testing and the test used for phenotyping. Antibodies are found in most of the clinically diseased animals, whereas in fecal shedders without clinical symptoms antibodies often are not detectable (Collins *et al*., 1991; Sweeney *et al*., 1995; Dargatz *et al*., 2001). Therefore, the result of a serological test depends on the age of testing (Nielsen and Toft, 2006) and the level of shedding (Sweeney *et al*., 1995; Whitlock *et al*., 2000; Dargatz *et al*., 2001). A causal relationship between the increased antibody synthesis and enhanced MAP shedding is still not established, but a higher lysis of macrophages in later stages of the disease is assumed to cause a higher antigen presentation to T- and B-cells and, therefore an increase in antibody secretion (Coussens, 2001).

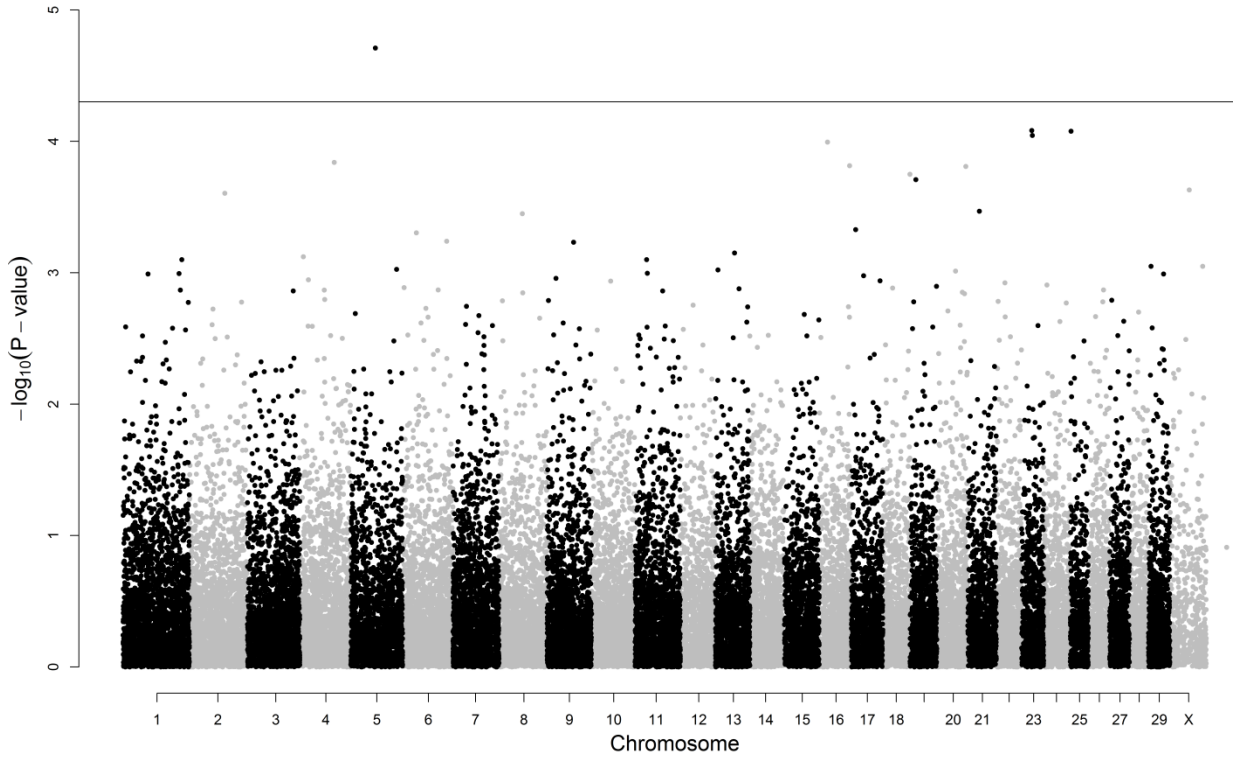

Figure 3 Association of single-nucleotide polymorphisms (SNPs) with *Mycobacterium avium* ssp. *paratuberculosis* (MAP)-infection in Holsteins phenotyped by ELISA$^{4+.}$ Manhattan plot of $-\log_{10}$ *(P*-values) with results of the whole-genome scan for an association of loci with *Mycobacterium avium* ssp. *paratuberculosis* ELISA$^{4+.}$ positive tested German Holstein cows. Positive is defined whenever one animal was positive in all four ELISA test results (ELISA^{4+}), regardless of fecal culture results The straight line at $-\log_{10}$ (5×10^{-5}) indicates a moderate significance level.

Table 5 *List of SNPs with P-values < 1.0e-04 of MAP ELISA positive tested Holsteins*

SNP	ARS-BFGL-NGS-10246	ARS-BFGL-NGS-91055	BTA-82554-no-rs	Hapmap58054-rs29014143	ARS-USMARC-Parent-DQ647186-rs290141	BTB-01513833	Hapmap42724-BTA-91683
Chromosome	5	25	15	3	11	20	23
Position	59694618	2315142	78627269	11199086	67033658	70628520	25956421
n	305	305	304	305	305	304	304
Minor/major allele	A/G	G/A	A/C	G/A	G/A	A/C	G/A
Allele frequency	0.10	0.09	0.45	0.42	0.42	0.49	0.49
Call rate	1	1	0.996721	1	1	0.996721	0.996721
$ELISA^{+}$ p=	1.43e-05*	1.89e-05*	8.56e-05	8.99e-05	8.99e-05	9.06e-05	6.63e-03
Odd ratio	3.26	3.82	0.47	0.47	0.47	0.48	0.60
$ELISA^{4+}$ p=	1.93e-05*	8.30e-05	6.33e-03	1.37e-03	1.37e-03	1.54e-04	8.94e-05
Odd ratio	3.04	3.22	0.58	0.52	0.52	0.46	0.42
FECAL p=	1.56e-03	6.83e-03	5.82e-02	2.87e-03	2.87e-03	2.56e-02	0.443
Odd ratio	2.41	2.34	0.73	0.61	0.61	0.70	0.88

* Moderately significant ($5x10^{-7} – 5x10^{-5}$)

ELISA positive is defined whenever an animals were positive in one ($ELISA^{+}$) or respectively in all four ($ELISA^{4+}$) of the four different ELISA tests.

No SNP with strong or moderate associations to the presence of MAP in feces could be found. However, we searched within Genbank for putative functional candidate genes on the basis of the SNPs with the strongest associations (Table 4) and obtained a list with more than 100 genes (within 1Mbp region of the different SNPs). Most of these genes appear to have a relation with immune response. None of these genes were found in previous published GWA studies (summarized in supplementary Table S1). Also there is no functional candidate gene described in the literature within 1 Mbp region of the SNPs in the present study (Settles *et al*., 2009; Minozzi *et al*., 2010; Pant *et al*., 2010; Kirkpatrick *et al*., 2011; Minozzi *et al*., 2012; van Hulzen *et al*., 2012). This supports once more the conclusion that paratuberculosis is probably affected by a large number of genes. Susceptibility loci for

complex disease identified in one population often cannot be replicated in other populations (Levinson *et al*., 2002). On the other hand it could be an effect of the unclear definition of the animal phenotype, because GWA-studies based on phenotype definition by different test methods identify different genes (Minozzi *et al*., 2012). Most GWA studies used milk or blood ELISA for phenotyping (Minozzi *et al*., 2010; Pant *et al*., 2010; Ruiz-Larranaga *et al*., 2010b; Kirkpatrick *et al*., 2011; Minozzi *et al*., 2012). ELISA may identify genes which are involved in the immune response to the agent, whereas bacterial culture of bacteria in feces or tissue will identify genetic loci, which are involved in the persistence of the MAP infection at different stages of the disease (Minozzi *et al*., 2012), the development of the granulomatous enteritis and the release of MAP into the feces. Another aspect is the design of the diverse studies. We used the case-control design, with MAP positive animals as cases and MAP negative controls as counterpart. The control animals match the cases with regard to environment and genetics that makes the case-control study more efficient than a cohort study of the same population. When using the results of antibody presence against MAP in blood, instead of using fecal culture results, two SNPs with a significant association to the detection of MAP antibodies ($ELISA^{+}$) were found on BTA 5 and 25. Considering only the animals with four positive ELISA test results ($ELISA^{4+}$) only the SNP on BTA 5 remains. Compared to the analysis of the fecal culture results, we got a totally different list of genes.

The two SNPs on BTA 5 and 25 are in regions where no genes have been identified so far. Considering all genes in the 1Mbp region around the SNPs as well as the function of these genes we can identify one gene of high interest on chromosome 25, the Interleukin 32 (*IL32*) gene (177.5 kb downstream of the putative SNP). Interleukins are proinflammatory cytokines, which are important in the signaling cascade of the immune response to a pathogen. The interaction and balance of the cytokines will influence the outcome of the immune response. IL32 has been implicated in inflammatory disorders, mycobacterium tuberculosis infections and inflammatory bowel disease in humans (Felaco *et al*., 2009). These facts make *IL32* both a biological and positional interesting candidate gene. To clarify this hypothesis further studies, for example sequencing of the interleukin, expressions studies and/or studies about the association of MAP status and the interleukins are necessary. To increase the evidence for the effects of genetic loci and the statistical power,

one approach is the joint analysis of two or more GWA datasets (Minozzi *et al*., 2012), but this approach requires that the same phenotyping method was used.

So we can conclude that several genes are located within 1 Mbp of the significant SNPs. One of these can be supposed as candidate gene for MAP susceptibility because of the function or as described in the literature. Compared with the literature, none of the significant markers found in this study were found in other GWA studies. Also within 1Mbp region of the significant SNPs no candidate genes for the susceptibility to MAP are described in the literature so far. The GWA study showed that different chromosomes and chromosomal regions are of interest by detection of MAP in fecal culture or the presence of antibodies against MAP in blood. One explanation for these different results could be the difference in sensitivity between the diagnostic tests. Another suggestion is, that numerous loci are involved in different stages of the disease, as expected. Therefore one of the greatest challenges for researchers in the field of paratuberculosis will be to define the important aspects of the multifactorial disease and then to find secure and continuous test methods for the different aspects of MAP infection.

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Supplementary material

To view supplementary material for this article, please visis http://dx.doi.org/10.1017/s1751731114001232

References

Aulchenko YS, Ripke S, Isaacs A and van Duijn CM 2007. GenABEL: a R package for genome-wide associations analysis. Bioinformatics 23, 1294-1296.

Chiodini RJ, van Kruiningen HJ and Merkal RS 1984. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. The Cornell Veterinarian 74**,** 218-262.

Collin MT, Sockett DC, Ridge S and Cox JC 1991. Evaluation of a commercial enzyme-linked immunosorbent assay for Johne`s disease. Journal of Clinical Microbiology 29, 272-276.

Collins MT 2003. Paratuberculosis: Review of present knowledge. Acta Veterinaria Scandinavica 44, 217-221.

Coussens PM 2001. Mycobacterium paratuberculosis and the bovine immune system. Animal Health Research Reviews 2, 141-161.

Dargatz DA, Byrum BA, Barber LK, Sweeney RW, Whitlock RH, Shulaw WP, Jacobson RH and Stabel JR 2001. Evaluation of a commercial ELISA for diagnosis of paratuberculosis in cattle. Journal of the American Veterinary Medical Association 218, 1163-1166.

Dreier S, Khol JL, Stein B, Fuchs K, Gutler S and Baumgartner W 2006. Serological, bacteriological and molecularbiological survey of paratuberculosis (Johne's disease) in Austrian cattle. Journal of Veterinary Medicine. B, Infectious diseases and veterinary public health 53, 477-481.

Donat K, Schau U, Soschinka A and Köhler H 2012. Herd prevalence studies of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in cattle using serological tests: opportunities, limitations and costs. Berliner und Münchner Tierärztliche Wochenschrift 125, 361-370.

Felaco P, Castellani ML, de Lutiis MA, Felaco M, Pandolfi F, Salini V, de Amicis D, Vecchiet J, Tete S, Ciampoli C, Conti F, Cerulli G, Caraffa A, Antinolfi P, Cuccurullo C, Perrella A, Theoharides TC, Conti P, Toniato E, Kempuraj D and Shaik YB 2009. IL-31: a newly-

discovered proinflammatory cytokine. Journal of Biological Regulators and Homeostatic Agents 23**,** 141-147.

Gonda MG, Kirkpatrick BW, Shook GE and Collins MT 2007. Identification of a QTL on BTA20 affecting susceptibility to *Mycobacterium avium* ssp. *paratuberculosis* infection in US Holsteins. Animal Genetics 38, 389-396.

Hinger M, Brandt H and Erhardt G 2008. Heritability estimates for antibody response to *Mycobacterium avium* subspecies *paratuberculosis* in German Holstein Cattle. Journal of Dairy Science 91, 3237-3244.

Kirkpatrick BW, Shi X, Shook GE and Collins MT 2011. Whole-genome association analysis of susceptibility to paratuberculosis in Holstein cattle. Animal Genetics 42, 149–160.

Kohl JL, Geisbauer E, Wassertheurer M, Revilla-Fernández S, Damoser J. Österreicher E, Dünser M., Kleb U and Baumgartner W 2012. Outcome of three commercial serum ELISAs and faecal detection of *Mycobacterium avium* subsp.p*aratuberculosis* in consecutive samples from cattle herd with low prevalence of paratuberculosis (Johne's disease). Transboundary and Emerging Disease 59, 197-207.

Küpper J, Brandt H, Donat K and Erhardt G 2012. Heritability estimates for *Mycobacterium avium* subspecies *paratuberculosis* status of German Holstein cows tested by fecal culture. Journal of Dairy Science 95, 2734-2739.

Levinson D, Holmans PA, Laurent C, Riley B, Pulver AE, Gejman PV, Schwab SG, Williams NM, Owen MJ, Wildenauer DB, Sanders AR, Nestadt G, Mowry BJ, Wormley B, Bauché S, Soubigou S, Ribble R, Nertney DA, Liang KY, Martinoloch L, Maier W, Norton N, Williams H, Albus M, Carpenter EB, deMachi N, Ewen-White KR, Walsh D, Jay M, Deleuze J-F, O'neill FA, Papadimitriou G, Weilbacher A, Lerer B, O'Donovan MC, Dikeos D, Silverman JM, Kendler KS, Mallet J, Crowe RR and Walters M 2002. No Major Schizophrenia Locus Detected on Chromosome 1q in a large Multicenter Sample**.** Nature 296, 739-740.

McKenna SLB, Keefe GP, Tiwari A, van Leeuwen J and Barkema HW 2006. Review: Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. The Canadian Veterinary Journal 47, 1089-1099.

Mendoza JL, Lana R and Diaz-Rubio M 2009. *Mycobacterium avium* subspecies *paratuberculosis* and its relationship to Crohn's disease**.** World Journal of Gastroenterology 15**,** 417-422.

Minozzi G, Buggiotti L, Stella A, Strozzi F, Luini M and Williams JL 2010. Genetic loci involved in antibody response to *Mycobacterium avium* ssp. *paratuberculosis* in cattle. PLoS ONE 5, e11117.

Minozzi G, Williams JL, Stella A, Strozzi F, Luini M, Settles ML, Taylor JF, Whitlock RH, Zanella R and Neibergs HL 2012. Meta-analysis of two genome-wide association studies of bovine paratuberculosis. PLoS ONE 3, e32578.

Montgomery GW and Sise JA 1990. Extraction of DNA from sheep white blood cells. The New Zealand Journal of Agricultural Research 33, 437–441.

Mucha R, Bhide MR, Chakurkar EB, Novak M and Mikula I 2009. Toll-like receptors TLR1, TLR2 and TLR4 gene mutations and natural resistance to *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle. Veterinary Immunology and Immunopathology 128, 381-388.

Muskens J, Barkema HW, Russchen E, van Maanen K, Schukken YH and Bakker D 2000. Prevalence and regional distribution of paratuberculosis in dairy herds in the Netherlands. Veterinary Microbiology 77, 253–261.

Nielsen SS and Toft N 2006. Age-specific characteristics of ELISA and fecal culture for purpose-specific testing for paratuberculosis. Journal of Dairy Science 89, 569-579.

Nielsen SS and Toft N 2009: A review of prevalences of paratuberculosis in farmed animals in Europe. Preventive Veterinary Medicine 88, 1-14.

Pant SD, Schenkel FS, Verschoor CP, You Q, Kelton DF, Moore SS and Karrow NA 2010. A principal component regression based genome wide analysis approach reveals the presence of a novel QTL on BTA7 for MAP resistance in Holstein cattle. Genomics 95, 176–182.

Pinedo PJ, Buergelt CD, Donovan GA, Melendez P, Morel L, Wu R, Langaee TY and Rae DO 2009a. Association between *CARD15*/*NOD2* gene polymorphisms and paratuberculosis infection in cattle. Veterinary Microbiology 134, 346-352.

Pinedo PJ, Buergelt CD, Donovan GA, Melendez P, Morel L, Wu R, Langaee TY and Rae DO 2009b. Candidate gene polymorphisms (BoIFNG, TLR4, SLC11A1) as risk factors for paratuberculosis infection in cattle. Preventive Veterinary Medicine 91, 189-196.

Purdie AC, Plain KM, Begg DJ, de Silva K and Whittington RJ 2011. Candidate gene and genome-wide association studies of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and sheep: A review. Comparative Immunology, Microbiology and Infectious Diseases 34, 197-208.

Ruiz-Larranaga O, Carrido JM, Iriondo M, Manzano C, Molina E, Koets AP, Rutten VPMG, Juste RA and Estonba A 2010a. Infection association between bovine *NOD2* polymorphisms and infection by *Mycobacterium avium* subsp. *Paratuberculosis* in Holstein-Frisian cattle. Animal Genetics 41, 652-655.

Ruiz-Larranaga O, Garrido JM, Manzano C, Iriondo M, Molina E, Gils A, Koets AP, Rutten VPMG, Juste RA and Estonba A 2010b. Identification of single polymorphisms in the bovine solute carrier family 11 member 1 (*SLC11A1*) gene and their association with infection by *Mycobacterium avium* subspecies *paratuberculosis*. Journal of Dairy Science 93, 1713-1721.

Settles M, Zanella R, McKay SD, Schnabel RD, Taylor JF, Whitlock R, Schukken Y, van Kessel JS, Smith JM and Neibergs H 2009. A whole genome association analysis identifies loci associated with *Mycobacterium avium* ssp. *paratuberculosis* infection status in US Holstein cattle. Animal Genetics 40, 655–662.

Sweeney RW, Whitlock RH, Buckley CL and Spencer P 1995. Evaluation of a commercial enzyme-linked immunosorbent assay for the diagnosis of paratuberculosis in dairy cattle. The Journal of Veterinary Diagnostic Investigation 7, 488-493.

van Hulzen KJE, Nielen M, Koets AP, de Jong G, van Arendonk JAM and Heuven HCM 2011. Effect of herd prevalence on heritability estimates of antibody response to

Mycobacterium avium subspecies *paratuberculosis.* Journal of Dairy Science 94, 992-997.

van Hulzen KJE, Schopen GCB, van Arendonk JAM, Nielen M, Koets AP, Schrooten C and Heuven HCM 2012. Genome-wide association study to identify chromosomal regions associated with antibody response to *Mycobacterium avium* subspecies *paratuberculosis* in milk of Dutch Holstein-Friesians. Journal of Dairy Science 95, 2740-2748.

Verschoor CP, Pant SD, You Q, Schenkel FS, Kelton DF and Karrow NA 2010. Polymorphisms in the gene encoding bovine interleukin-10 receptor alpha are associated with *Mycobacterium avium* ssp. *paratuberculosis* infection status. BMC Genetics 11, 23.

Whitlock RH, Wells SJ, Sweeney RW and VanTiem J 2000. ELISA and fecal culture for paratuberculosis (Johne´s disease): sensitivity and specificity of each method. Veterinary Microbiology 77, 387-398.

10. Additional files

Supplementary Table S1: MAP associated loci from different genome wide association studies in dairy cows

The table shows the so fare identified gene loci with an association to the MAP status for dairy cows and putative candidate genes from regions within 1 Mb of the SNPs from different genome wide association studies.

Breed	Test method for MAP status	Genomic region associated with MAP and putative candidate genes within 1 Mb of the SNPs	Author
US Holsteins	Culture from feces and necropsy tissue	Tissue: BTA 3 (Pos.: 110682051bp) Genes: 1Mb: *FOXJ3*, *EDN2*, *CTPS*, *CITED4*, *NFYC* Shedding: BTA 9 (Pos.: 813310bp) Genes: 1Mb = none	Settles *et al*., 2009
Italian Holsteins	ELISA (ID-screen®)	BTA 9 (Pos.: 46362363bp) Genes: *PRMD1* or *Blimp1* BTA 12 (Pos.: 69663832bp, 695599639bp) Genes: ATP-binding cassette, subfamily C (*CFTR/MRP*), member 4 protein (*ABCC4*) BTA 11 (Pos.: 89695127bp) Genes: *E2F6*, *PQLC3*, *C2o50*, *KCNF1*, *PDIA6*, *ATP6V1C2*, *NOL10*, *ODC1*, *HPCAL1*	Minozzi *et al*., 2010
US Holsteins	Milk or blood ELISA (Pourquier®)	BTA 1 (Pos.: 40758982bp) Genes: *TUBA3D* BTA 5 (Pos.: 14416892bp, 14500783) Genes: *CCDC59, TMTC2* BTA 5 (Pos.: 37186379) Genes: *FAM113B, AMIGO2, SLC38A4, SLC38A2, SLC38A1, SFRS2IP, ARID2* BTA 5 (Pos.: 41512973bp) Genes: *PRICKLE1, PPHLN1, ZCRB1, YAF2, GXYLT1v* BTA 5 (Pos.: 87137388bp, 88577519bp, 88838035bp) Genes: *TMTC1, OVCH1, ERGIC2, FAR2, TM7SF3, CCDC91, PTHLH, KLHDC5, RPS35, FGFR10P2, PPFIBP1, RNTL2, STK38L, MED21, ITPR2* BTA 6 (Pos.: 108202550bp, 108228418bp) Genes: *CRMP1, EVC, EVC2, STX18, STK32B, MSX1, CYTL1* BTA 7 (Pos.: 17929919bp, 17957376bp, 17983797bp, 19746463bp)	Pant *et al*., 2010

		Genes: *IRF1, IL5, IL13, IL4* BTA7 (Pos.: 83397046bp) Genes: SSBP2, ATG10, XRCC4 BTA 10 (Pos.: 51097099bp, 52944489bp, 52966013bp) Genes: *NARG2, ANXA2, FOXB1, ADAM10, GRINL1A,BNIP2, GTF2A2, LIPC, TCF12, MYO1E, CCNB2, RNF111, AQP9* BTA 10 (Pos.: 60612527) Genes: GLDN, *SCG3, LYSMD2, TMOD2, TMOD3, LEO1, GNB5, MYO5C, AP4E1, TRPM7, USP50, USP8, GABPB2, HDC, SLC27A2* BTA 11 (Pos.:30601961bp) Genes: *PRKCE, EPAS1, ATP6V1E2, PIGF, CRIPT, SOCS5, MCFD2, TTC7A, EPCAM, MSH2, KCNK12*, *MSH6, FBXO11* BTA 11 (Pos.: 53982484bp, 54033703bp) Genes: *ANGPT1, RABL4, RSPO2, EIF3E, TTC35, TMEM74, TRHR*	
US Holsteins	Fecal culture or ELISA (Pourquier®)	BTA 3, BTA 6, BTA 14, BTA16, BTA 17, BTA 20, BTA 23, BTA 24, BTA 26	Kirkpatrick *et al*., 2011
US and Italian Holsteins from the studies: Settles *et al*., 2009 and Minozzi *et al*., 2010	ELISA (ID-screen®) or Tissue culture	BTA 1 (Pos.: 113617698bp, 113855358bp) Genes. *SSRG*, *SCL33A1*, *KCNAB1*, *GMPS* BTA 1 (Pos.: 3083368bp, 3083498bp) Genes. *SOD1*, *HUNK*, *SFRS15* BTA 6 (Pos.: 22013011bp) Genes: 1Mb = none BTA 7 (Pos.: 40664184bp) Genes: 1Mb = none BTA 9 (Pos.: 46362363bp) Genes: *PRMD1* or *Blimp* BTA 12 (Pos.: 69663832bp, 695599639bp) ATP-binding cassette, subfamily C (*CFTR/MRP*), member 4 protein (*ABCC4*) BTA 13 (Pos.: 659777384bp) Genes: 1Mb = none BTA 15 (Pos.: 66161046bp) Genes: *LDLRAD3, PAMR1*, *CACNA1B*, *COMMD9* BTA 16 (Pos.: 86317804bp) Genes: 1Mb = none BTA 21 (Pos.: 33135132bp)	Minozzi *et al*., 2012

		Genes: 1Mb = none BTA. 22 (Pos.: 56087082bp) Genes: 1Mb = none BTA 23 (Pos.: 34108529bp) Genes: 1Mb = none BTA 25 Genes: 1Mb = none	
Dutch Dairy cattle with at least 75% Holstein-Friesian genes	Milk ELISA (Pourquier®)	BTA 4 (Pos. 51425144bp) Genes: *DLD*, *Q28899*, *LAMB4*, *Q6Q146*, *PNPLA8*, *LOC784535*, *Q2YDK7*, *Q17QP5*, *EPDR1* BTA 15 (Pos.: 60 257030bp-60733532bp) Genes: *KCNA4*, *FSHB*, *LOC787432*, *A5PJ77*, *CK046* BTA 15 (Pos.: 22546064bp) Genes: *A2VDX5*, *IRX5* BTA 28 (Pos.: 11823511bp-11839054bp) Genes: *ACM3*, *Q3SX15*, *NSBTAG00000018960*, *ZNF25*, *ZNF334*, *LOC534200*, *ENSBTAG00000013592*, *BMS, RET*, *CSGALNACT*, *Q5E9S7*	van Hulzen *et al*., 2012

6. GENERAL DISCUSSION AND CONCLUSION

ParaTB or Johne's disease caused by *Mycbacterium avium* ssp. *paratuberculosis* is an ubiquitary chronically mucosal enteritis. The present study focused on economic losses and the genetics of MAP infection in German Holstein cows. Therefore, we used for the first time a large dataset of 11 285 fecal culture tested German Holstein cows to estimate the heritability for MAP infection and quantify economic losses in connection with MAP infection. Furthermore, associations were estimated between the potential candidate gene *NOD2* and MAP infection as well as between a whole genome scan and MAP infection. For the association tests additional results of four commercial ELISA tests were available beside the fecal culture test result.

Structures of MAP status within the farms

All animals of the studies originate from 15 farms in Thuringia. The prevalence rates ranged between 2.8% and 25.4% (Ø 9.8%) with the exception of one farm with a prevalence of 67.9% (including this farm Ø 13.7%). All farms participate voluntary in the "Paratuberculosis Control Program in Thuringia Cattle Herds" organized by the Thuringia Animal health service (Donat, 2009). In this connection all female cows older than 24 months are tested for MAP by fecal culture in 2008 and 2009 whereas it is recommended to cull all positive tested cows according to Sweeney *et al.* (2012). The farm with the prevalence of 67.9% did not react with culling of positive tested animals so far, explaining the high prevalence rate. However, other farms participated for a longer period in the program. A later study within the same voluntary eradication program of Thuringia concerning nearly the same farms showed similar results (Donat *et al.*, 2012). In this study the animals were tested with the same fecal culture method in the following years 2009 and 2010. Thereby, the results showed in tendency an improvement of the single prevalence rates, which are between 1.7% and 19.9% (Ø 7.3%) in 14 farms and with additional two farms with rates of 45.0% and 58.8% (Ø 12.98%). These results lead to the conclusion that the strictness of culling and management factors seems to be responsible for the different prevalence rates and therefore for the success of the program. This is confirmed by other studies, which also highlighted the role of management, especially in replacement, calving and hygiene practice, to contain the infections with MAP (Raizman *et al.*, 2004; McKenna *et al.*, 2006; Collins *et al.*, 2010).

Nevertheless, based on long term considerations the goal should be to eradicate the disease to avoid the culling of animals.

Heritability

Due to the lack of therapy or sufficient prevention (e.g. vaccination), breeding strategies against MAP come into focus. It is a basic requirement, that a parameter is heritable, if it will be used for breeding purposes. A few traits in breeding programs have a low heritability, like parameters of fitness and especially fertility (e.g. number of offspring, calving interval, non return rate) but also disease traits (e.g. mastitis [h^2 = 0.04 – 0.08; Urioste *et al.*, 2012; Pritchard *et al.*, 2013], in Germany indirectly measured by somatic cell count with h^2 = 0.16 [VIT, 2013]). The heritability for MAP infection, phenotyped by ELISA test, were denoted between 0.031 and 0.16 depending on the manufacturer, breed, number of animals, source material (serum or milk) and statistical model used (Mortensen *et al.*, 2004; Gonda *et al.*, 2006; Hinger *et al.*, 2008; Attalla *et al.*, 2010; van Hulzen *et al.*, 2011). Even if fecal culture is more sensitive and specific than serum or milk ELISA, it is more practicable to use the manageable and cost efficient ELISA test for diagnosis, especially for herd diagnosis. For the same reasons the majority of studies about paratuberculosis deal with ELISA test results. So far, only Gonda *et al.* (2006) have estimated heritability for MAP infection of 0.153 using 3 464 fecal cultures tested cows, giving a hint of a higher genetic background. In the present study we could confirm this hypothesis in a larger dataset of animals. Thereby, depending on the statistical model and dataset (excluding the farm with the high prevalence rate and excluding sires with more than four daughters), the estimated heritability ranged between 0.157 and 0.228. In all cases the heritability was higher in the sire model than in the animal model, which is in contrast to the assumption of van Hulzen *et al.* (2011), but in accordance with Ramirez-Valverde *et al.* (2001). Van Hulzen *et al*. (2011) presumed that due to possible intrauterine MAP infections of calves, the animal model will estimate a higher genetic variance. In contrast Ramirez-Valverde *et al.* (2001) assumed that the additive genetic variance will be overestimated in the sire model in cases of low numbers of daughters per sire. When comparing the results in the present study of the sire model of the whole dataset (h^2=0.195) and the dataset considering only sires with more than four daughters (h^2=0.175),

we could confirm the results of Ramirez-Valverde *et al.* (2001). In conclusion, the higher heritability estimated on the basis of fecal culture, makes this diagnostic method a useful tool for further genetic analysis. Furthermore, these heritabilities reach values which are comparable with traits like somatic cell count, litter size in pigs, or eggs per hen, which are already included in breeding programs in cattle, pigs and chicken, respectively. These traits reached positive developments since the inclusion in the respective breeding program, showing the potential for the inclusion of the prevalence to MAP in breeding programs in cattle. Another aspect is the great effort to consider parameters for health and fitness in different breeding programs for dairy cattle. There is an optimistic prospect to establish a breeding value for fitness in Germany despite low heritability. The high heritability for MAP infection phenotype by fecal culture is a good basis for a possible integration of this parameter into breeding values. However, in this case other aspects, e.g. economical relevance or possible positive or negative correlations with other traits, have to be considered. Beside that it is to mention, that the success of a genetic selection depends on the kind of selection strategy. Van Hulzen *et al.* (2014) demonstrated that the response for selection is larger using sire selection based on estimated breeding values than for "classical" dam selection based on phenotype definition. Certainly, the success, which means the time for eradication, depends also on prevalence rates, selection intensity, and the accuracy of breeding values for sires.

Differences in productivity between MAP infected and non-infected herds

After estimating the genetic background of MAP infection by fecal culture we analyzed the influence of the MAP infection on milk production traits, like milk yield, milk fat and milk protein, the influence on milk kilograms per day of life, interaction between farm or lactation number and fecal test result, as well as on calving interval. From the total 11 285 fecal tested animals, milk recording data (305-days) of 9 367 animals from 14 farms were analyzed. Additional to the fecal test results (dataset 1) we used subsets of 870 animals to analyze the influence to the above mentioned parameters, whereas the animals were additionally tested with four different ELISA tests (dataset 2, not shown in the second study of the present thesis in chapter 3).

The distribution of animals per number of lactation per test is summarized in Table 4. Both "farm" and "lactation number" have a significant ($p \leq 0.001$) influence on all parameters considered. A significant decrease in milk production of 2.4% for fecal culture positive animals could be observed and confirmed the results of reduction between 2 – 6.8% (Hendrick *et al.*, 2005a; Sorge *et al.*, 2010).

Table 4: Distribution of animals per number of lactation. Dataset 1: in total 9 505 animals, tested for MAP by fecal culture with 305-day lactation yield. Dataset 2: a subset of Dataset 1 860 animals were additional tested with four different ELISA tests.

Lactation number	MAP-test result	Dataset 1	Dataset 2				
		Fecal culture	Fecal culture	ELISA A	ELISA B	ELISA C	ELISA D
1	neg	3522	134	185	178	171	178
	pos	412	80	27	36	40	36
2	neg	2363	140	214	204	204	207
	pos	344	113	29	48	47	46
3	neg	1257	116	147	141	140	140
	pos	208	59	19	30	32	35
≥4	neg	1224	164	196	182	181	188
	pos	175	64	28	41	44	40
n=		9505	870	845	860	859	870

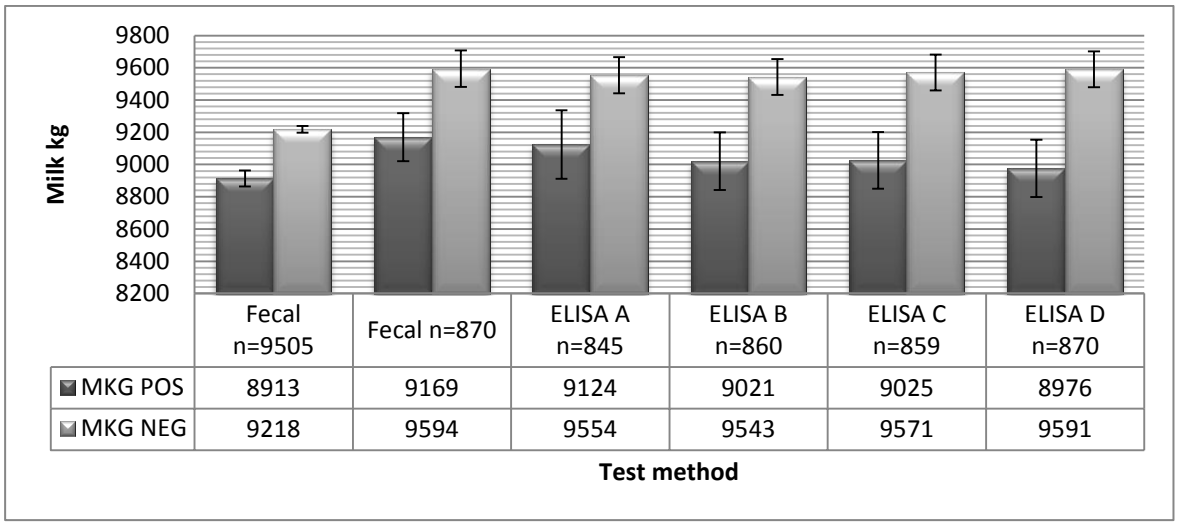
Also a reduction of fat and protein kilograms could be observed in the present study. The significances of the reduction in milk parameter in dataset 2 is shown in Table 5. The reduction in milk kilograms (up to 6.4%, shown in Figure 2), fat kilograms (Figure 3) and protein kilograms (Figure 4) was higher in ELISA positive tested cows than concerning the fecal culture results and therefore in contrast to the results described in the literature (Hendrick *et al.*, 2005b; Gonda *et al.*, 2007). In

Table 5: Significances of the influence of the MAP status tested by different tests, the farm and the number of lactation for the milk traits milk in kg, fat in kg and protein in kg of the 305-day.

		305-day lactation		
Dataset	Parameter	milk	fat	protein
1 and 2	farm	***	***	***
	lactation number	***	***	***
1	fecal culture	***	***	***
	Interaction farm x fecal culture	*	*	*
2	fecal culture	**	*	**
	ELISA A	*	*	**
	ELISA B	**	**	**
	ELISA C	**	**	**
	ELISA D	**	**	**

n.s. = not significant
* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

both above mentioned publications the prevalence rates using fecal cultural tests are lower than using ELISA-tests in contrast to our study, which could be a possible explanation for the differing results. An other reason could be the usage of a high sensitive diagnostic method, which leads to a detection in an earlier stage of MAP-infection and with this to a less milk yield depression in fecal positive cows (Aly *et al*., 2010). However, milk fat- and protein-percentage did not differ between positive and negative animals, leading to the conclusion that the decrease in fat and protein yield will be in proportion of milk yield and the milk composition will be unchanged. In general the described results in literature of reduced milk production in positive animals are heterogeneous and in a wide range (Nordlund *et al.,* 1996; Hendrick *et al.*, 2005b; Gonda *et al.*, 2007; Tiwari *et al.* 2007, Sorge *et al.*, 2010).



	Fecal n=9505	Fecal n=870	ELISA A n=845	ELISA B n=860	ELISA C n=859	ELISA D n=870
MKG POS	8913	9169	9124	9021	9025	8976
MKG NEG	9218	9594	9554	9543	9571	9591

Figure 2: LS means for positive and negative tested cows by test result for the average 305-day milk yield in kg.

Beside the influence of different used test methods, a main reason therefore could be the significant interaction between farm and test results shown in our study. Furthermore, in the second study of the present thesis (chapter 3) we demonstrated that farms with a lower herd prevalence (<12%) showed lower differences in milk reduction of positive animals, than farms with a high herd prevalence, whereas the influence of prevalence rate was not significant. This is in accordance to the study of Donat *et al*. (2014). They found a distinct tendency in milk yield reduction of fecal culture positive cows from herds with medium or high within-herd prevalence, whereas the difference in milk yield of fecal positive cows from

herds with low prevalence was marginal. The observed influence on milk yield of the within-herd prevalence was also not significant.

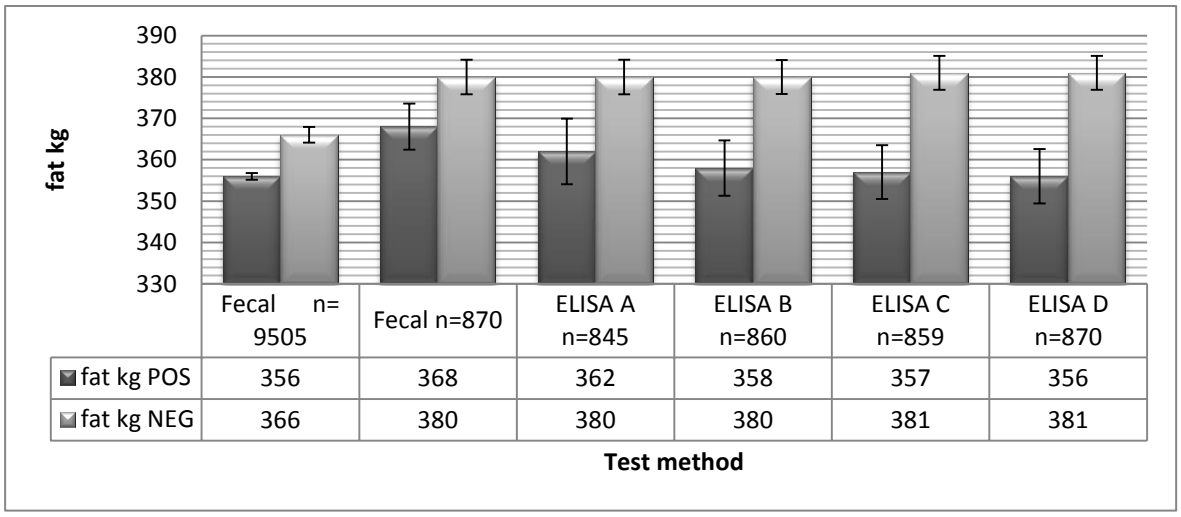


Figure 3: LS means for positive and negative tested cows by test result for the average 305-day fat in kg.

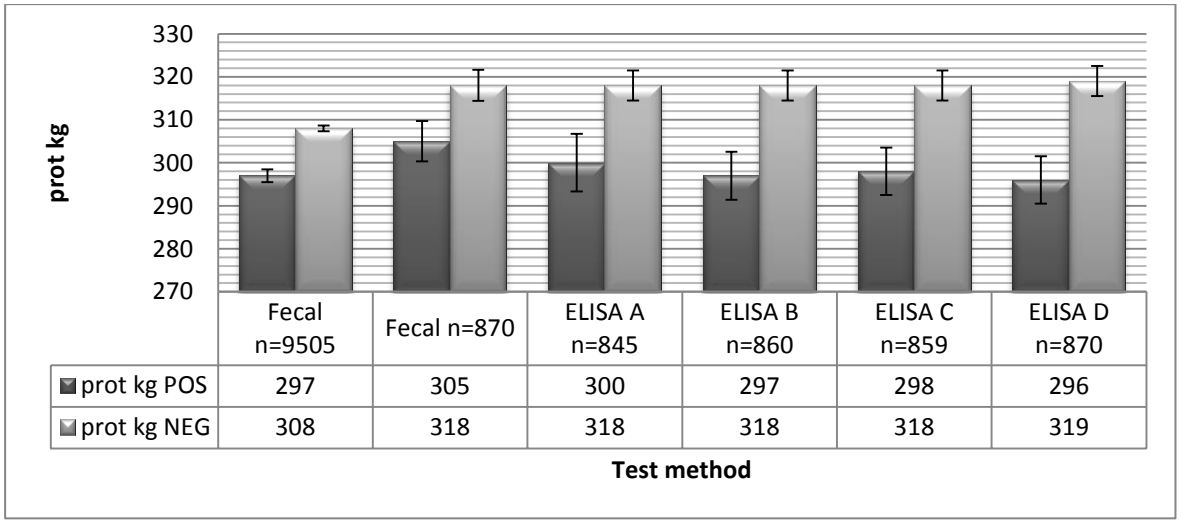


Figure 4: LS means for positive and negative tested cows by test result for the average 305-day milk protein in kg.

In the present study the level of milk production had no influence on the differences between positive and negative animals. Considering the milk kilograms per day of live there is no difference between positive and negative cows within the first lactation. In the later lactations the difference between the positive and negative animals increased and is highly

significant. It is assumed that this effect is a cumulative effect of the reduced milk production per lactation of MAP infected animals and the increased calving interval of the positive tested cows vs. negative ones.

The calving interval or the days open are often used parameters for reproductive efficiency. In the present study we used the calving interval and found a two day shorter calving interval in fecal culture negative animals compared to the positive tested animals ($p = 0.678$). Neither the interactions between fecal test result and farm ($p = 0.331$) nor the interactions between fecal test result and lactation number ($p = 0.661$) show a significant influence on calving interval and therefore we agree with the results of the study of Ansari-Lari *et al.* (2012). They showed nearly significant differences in calving interval between positive and negative tested herds. Other studies reported no significant difference between positive and negative cows (Raizman *et al.*, 2009) or reported a significant increase in days open in ELISA positive cows, but no significant differences in fecal tested cows (Johnson-Ifearulundu *et al.*, 2000). Another study reported that cows with strong positive ELISA test results showed fewer days open than cows with negative ELISA test results (Lombard *et al.*, 2005). These conflicting results may be on one hand depending on the different test methods used, on the other hand we could demonstrate a significant interaction between farm and MAP status for the first calving age ($p \leq 0.05$).

The results of MAP infection to production traits underline the economic importance of eradication of MAP infections in dairy cattle for an economic milk production. Unfortunately, we could only estimate a part of the real losses in dairy cattle because of the lack of data concerning culled animals as result of MAP infection, and costs for higher management factors and hygiene measures in affected farms. However, the economic losses also concern the beef cattle industry, where annual losses were estimated between 25 US$ and 235 US$, because of reduced calving proportions, higher calf mortality, lower weaning weight and higher veterinary costs (Bhattarai *et al.*, 2013).

Candidate gene NOD2 and whole genome study

After clarifying the negative influence of MAP infection on economic parameters in dairy cattle and the confirmation of a genetic background, we searched for potential candidate genes. Numbers of genetic loci associated with MAP infection were described in literature (reviewed by Purdi *et al.*, 2011). Especially, genes associated with Crohne's disease in humans were considered as candidate genes for MAP infection, because of the discussed relationship between both diseases. Furthermore, the function of the NOD2 protein makes the gene as a potential functional candidate gene of interest. NOD2 identifies parts of the mycobacterial cell wall (Oh *et al.*, 2005) and induces the activation of the NF-κB proinflammatory signaling pathway (Ogura *et al.*, 2001), which is involved in the regulation of different genes, like the cytokines or interleukins, which are part of the immune response. To exclude possible stratification factors, we performed a case control study with in total 324 animals, tested by fecal culture. Every positive tested animal older than 24 months (to give consideration to the long incubation time of the disease), was matched to a negative one from the same farm, with the same sire and at the same age or older. These parameters were chosen to ensure that the positive and negative animals were exposed to the same infection stress and grown up in the same environment. As mentioned before, this particular farm has a high significant influence. Half siblings or, if possible, twins were used to get preferable similar genetic background of the animals. Additionally, ELISA test results of all animals tested by four different fabrics were available.

In a well examined gene like the *NOD2* it is still possible to identify further SNPs. Thus, in total we identified 11 SNPs within the *NOD2* gene, whereas five of these SNPs were identified in previous studies by Hinger (2009), Taylor *et al.* (2006), and Ruiz-Larrañaga *et al.* (2010b). Two of these SNPs were described with a significant influence on the MAP status of the animals, one in exon 4 tested by ELISA (Hinger, 2009) and one in the 3'UTR tested by fecal culture or PCR or ELISA (Ruiz-Larrañaga *et al.*, 2010b). Four of the SNPs were considered in our case control study because of their position in exon (SNP g.521, exon 4) or 3'UTR region (g.1633, g.2083 and g.2580). Thereby, we could not confirm the influence of the SNP g.2083 in the 3'UTR described by Ruiz-Larrañaga *et al.* (2010b) by the case control study. However, the significant influence of the SNP g.521 in exon 4 (Hinger, 2009) to the

MAP status of the animals could be confirmed (p = 0.0056). Hinger (2009), who studied German Holsteins from the same farms and with the same structure of case and control animals as in the present study, identified a higher frequency for the G allele in uninfected animals than in the infected ones. This is contrary to the present study, where the frequency of the G allele was higher in infected animals than in uninfected. Furthermore, in a subset of 99 ELISA positive and 99 matching ELISA negative animals from the total 324 animals we could not confirm the association of the difference in allele frequencies and the test result. Associations detected with alleles with low frequency should be interpreted with caution, which is one explanation for varying results. Another possibility could be that the SNP is in linkage disequilibrium to a hidden and so far unknown causative polymorphism, and different haplotypes between the studied SNPs and the causative SNP may exist.

No other associations could be determined with polymorphisms of *NOD2* and the MAP infection status of the animals. Also, the more frequent C allele on position c.2197 in MAP infected Brahman-Angus cattle than in not infected reported by Pinedo *et al.* (2009c) could not be confirmed in the present study. As expected and in agreement with Taylor *et al.* (2006), the German Holstein cows showed no variability at this position and were monomorph for the T allele. The SNP in *NOD2* identified by Pinedo *et al.* (2009c) seems to be special for *Bos indicus*.

Also, the analysis of the haplotype frequencies of the four analyzed SNPs (g.521 in exon 4; g.1633, g.2083 and g. 2580 in 3'UTR) showed no association to the MAP infection status of the animals. Thereby, most frequent haplotypes, in order SNP-No.: 1, 2, 3, and 4, were GACG with a frequency of 41.0%, GCCG with a frequency of 37.9% and GCTA with 13.3%. The haplotype frequencies are unchanged when only considering SNPs within the 3'UTR (SNP-No. 2, 3 and 4). Some of the SNPs show high linkage disequilibrium but using the confidence interval (Gabriel *et al.*, 2002) no highly frequent haplotype block could be defined. No other comparable results from the literature were available, demonstrating the lack of haplotype studies in this context. As it is often meaningful to implicate the complete variability of a gene using haplotypes, like described in further studies e.g. caseins (Küpper *et al.*, 2010), further research is needed.

By proceeding development in gene technology the costs for the usage of SNP-chips are further reduced and therefore this technology today is a useful tool for studies with higher numbers of animals. Also the handling and the accuracy of the test are reasons that classical QTL and to part candidate gene studies are replaced by genome wide association studies by SNP-chips. Assuming that paratuberculosis is influenced polygenetic, we used again the case-control study based in fecal culture test results for the GWA study. The control animals match the cases with regard to environment and genetics (Kirkpatrick *et al*., 2011) that makes the case-control study more efficient than a cohort study of the same population. Next to the GWA study fecal culture positive vs. fecal culture negative animals we used subsets based on the ELISA test result. Regardless of the fecal culture results, ELISA positive is defined whenever one animal was positive in one of the four different ELISA test results and ELISA^{4+} positive is defined whenever one animal was positive in all four different ELISA tests. Beside the two subsets presented in the fourth study of the presented thesis (Chapter 5) we analyzed also the subsets ELISA^{2+} and ELISA^{3+} where positive is defined whenever one animal was positive in two respectively in three of the four different ELISA test results (Table 6). In total, we could find no SNP with strong or moderate associations to the presence of MAP in feces, whereas in the subsets (ELISA^{+}, ELISA^{2+}, ELISA^{3+}, and ELISA^{4+}) four SNPs with a significant association to the presence of MAP antibodies in serum could be detected on chromosomes 5, 20, 23 and 25 (Table 7). All the associated markers are in regions with genes unidentified thus far. According to other GWA studies (e.g. Settles *et al*., 2009; Minozzi *et al*., 2010) we searched within GenBank (http://www.ncbi.nlm.nih.gov/genbank/) for putative functional candidate genes within 1Mbp region of the different SNPs. Thereby, numerous genes with function in immune response could be identified whereas none of these SNPs could confirm or matched with SNPs from a

Table 6: Number of positive ELISA test results for MAP in comparison to the fecal culture results. ELISA positive is defined whenever animals were positive in one (ELISA^{+}), two (ELISA^{2+}), three (ELISA^{3+}) or respectively four (ELISA^{4+}) of the four different ELISA test results.

		fecal POS	fecal NEG
		n=152	n=153
ELISA^{+}	POS	94	27
	NEG	58	126
ELISA^{2+}	POS	91	22
	NEG	61	131
ELISA^{3+}	POS	87	19
	NEG	65	134
ELISA^{4+}	POS	84	17
	NEG	68	136

previous GWA study (Settles *et al*., 2009; Minozzi *et al*., 2010; Pant *et al*., 2010; Kirkpatrick *et al*., 2011; Minozzi *et al*., 2012; van Hulzen *et al*., 2012). Three genes of high interest within 1Mbp region of the significant SNPs (Table 7) could be identified: one on chromosome 25, the interleukin 32 (*IL32*) gene (177.5 kb downstream of the putative SNP) and two genes on chromosome 23, the *IL17A* gene (2,6kb downstream) and the *IL17F* gene (46kb downstream). On BTA 5 no functional candidate gene could be found within 1Mbp of the significant SNP.

Table 7: List with p-values of the most associated SNPs with MAP ELISA positive tested Holsteins. ELISA positive is defined whenever animals were positive in one ($ELISA^{+}$), two ($ELISA^{2+}$), three ($ELISA^{3+}$) or respectively four ($ELISA^{4+}$) of the four different ELISA test results, regardless of fecal culture results.

SNP	**ARS-BFGL-NGS-10246**	**ARS-BFGL-NGS-91055**	**ARS-BFGL-NGS-100992**	**BTB-01513833**	**BTA-85226-no-rs**	**BTA-62789-no-rs**	**BTA-82554-no-rs**	**ARS-USMARC-Parent-DQ647186-rs290141**	**Hapmap58054-rs29014143**	**ARS-BFGL-NGS-70466**	**Hapmap42724-BTA-91683**
Chromosome	5	25	23	20	4	4	15	11	3	18	23
Position	59694618	2315142	24344384	70628520	21022962	83890518	78627269	67033658	11199086	65957749	25956421
N	305	305	300	304	302	304	304	305	305	305	304
Minor/major allele	A/G	G/A	A/G	A/C	G/A	G/A	A/C	G/A	G/A	A/G	G/A
Allele frequency	0.104918	0.085246	0.328333	0.490132	0.034768	0.300987	0.445724	0.42459	0.42459	0.354098	0.491776
Call rate	1	1	0.983607	0.996721	0.990164	0.996721	0.996721	1	1	1	0.996721
ELISA+ p=	**1.43e-05***	**1.89e-05***	7.24e-04	9.06e-05	6.13e-04	8.92e-04	8.56e-05	8.99e-05	8.99e-05	2.60e-04	6.63e-03
Odd ratio	3.235989	3.817136	1.805889	0.483214	5.590246	1.791729	0.467539	0.467702	0.467702	0.487902	0.596294
$ELISA^{2+}$ p=	**1.70e-05***	**3.62e-05***	1.37e-04	**3.46e-05***	2.04e-04	2.38e-04	3.01e-04	2.54e-04	2.54e-04	8.94e-05	2.78e-04
Odd ratio	3.136062	3.525592	1.94423	0.445226	6.325598	1.903166	0.488704	0.482552	0.482552	0.44412	0.537048
$ELISA^{3+}$ p=	**3.29e-05***	1.04e-04	**4.35e-05***	2.38e-04	5.15e-04	4.15e-04	2.15e-03	4.74e-04	4.74e-04	6.53e-04	2.78e-04
Odd ratio	3.018911	3.251634	2.056795	0.477503	5.302188	1.873004	0.536824	0.483075	0.483075	0.484807	0.454408
$ELISA^{4+}$ p=	**1.93e-05***	8.30e-05	8.19e-05	1.54e-04	1.13e-03	1.43e-04	6.33e-03	1.37e-03	1.37e-03	1.77e-04	8.94e-05
Odd ratio	3.04211	3.217037	1.986525	0.460832	4.460689	1.945302	0.578364	0.516151	0.516151	0.438872	0.415848
FECAL p=	1.56e-03	6.83e-03	7.89e-02	2.56e-02	4.50e-02	3.01e-02	5.82e-02	2.87e-03	2.87e-03	0.145368	0.443
Odd ratio	2.405938	2.344816	1.358093	0.698062	2.668839	1.463655	0.730075	0.60511	0.60511	0.78148	0.877839

** = strongly significant ($<5x10^{-7}$), * = moderately significant ($5x10^{-7} – 5x10^{-5}$)

Interleukins are proinflammatory cytokines and important in the signaling cascade of the immune response to a pathogen. The outcome of the immune response is influenced by the interaction and balance of the cytokine. The *IL17* family contains six members (*Il17A* to *IL17F*). *IL17A* and *IL17F* share 50% sequence homology and both can exist as biologically active homodimer (IL17AA and IL17FF) ore heterodimers (*IL17AF*)- *IL17A* and *IL17F* induces the expression of pro inflammatory chemokines and cytokines (e.g. CXXL1, CXCL8, CXCL10, TNF-α, IL1 or IL6), which are involved in the regulation of local tissue inflammation (Pappu *et al*., 2010). In vivo studies in mice demonstrate that IL17A and IL17F play roles in the response to local gram-negative and gram-positive bacterial infections such as Mycobacterium tuberculosis; furthermore IL17A is important in memory response to these pathogens. Also, IL17 in previous study deals with the connection of several interleukins and Johne's disease in different species. Shu *et al*. (2011) reported a significant up regulation of mRNA expression of *IL17A* in antigen stimulated mesenteric lymph nodes from infected animals. An up regulation of *IL17* was also described by Robinson *et al*. (2011) in MAP infected red deer. In humans, increased production of IL17 within inflammed mucosa from inflammatory bowel disease, such as Crohn's disease (Fujino *et al*., 2003), was observed. Also, *IL32* has been implicated in inflammatory disorders, mycobacterium tuberculosis infections and inflammatory bowel disease in humans (Felaco *et al*., 2009). These facts make the *IL17A* and *IL17F*, as well as *IL32* functional and positional candidate genes, but there are other SNPs which are included on the SNP chip and which are located closer to these genes or within these genes. As they show no significant associations to MAP other conclusion could be that there is probably no functional association between *IL32*, *IL17A* as well as *IL17B* and the antibody response to MAP. However, the inconsistent results compared with other GWA studies concerning MAP infection leads ones more to the conclusion that paratuberculosis is not affected by just one gene. Otherwise, different test methods identify different types of genes (Minozzi *et al*., 2012) and the animals of the different studies were phenotyped by different test methods. Thus, the majority of GWA studies used milk or serum ELISA for classifying the animals into MAP infected or uninfected (Ruiz- Larrañaga *et al.*, 2010b; Minozzi *et al*., 2010; Pant *et al*., 2010; Kirkpatrick *et al*., 2011; Minozzi *et al.*, 2012), whereas ELISA may identify genes which are involved in the immune response to the agent. Bacterial culture of bacteria in feces or tissue will identify gene loci, which are

involved in the persistence of the MAP infection at different stages of the disease (Minozzi *et al.*, 2012). Also notable again are the designs of the diverse studies. So we used the typical case-control design, with MAP positive animals as cases and MAP negative controls as counterpart (Kirkpatrick *et al*., 2011).

To give our candidate gene analysis of the *NOD2* more evidence, we used data of the Illumina SNP50 BeadChip and looked for the SNPs on chromosome 18 in the region around *NOD2*. Within the gene there are no SNPs on the chip. The next flanking SNPs seem to be 47 765 kbp upstream and 150 285 kbp downstream. Both SNPs showed no association to the MAP status of animals. Considering the Affymetrix 25k Panel so the next SNP seems to be 1 577 Mbp upstream from the *NOD2* gene (http://www.livestockgenomics.csiro.au/) and therefore not an informative maker for the *NOD2* gene.

Problem of phenotyping

One main question concerning ParaTB or MAP-infection is still unclear: What is the intention of implementing genetic makers for breeding against MAP? Animals which are resistant to the disease or animals are unsusceptible for MAP. When considering the previously discussed studies, it always comes back to the main problem: the method for phenotyping MAP infection. There are not only differences between the single tests, but also within the same test method. In the dataset of the case-control study the differences in the number of positive / negative animals between fecal test results and ELISA test results (Table 6) within the same population show the diagnostic complexity and therefore the difficulty in defining the phenotype of the animals in a correct way. Even the differences between the four ELISA test results (Table 8) reflect the same problem. The differences between the fecal test results and ELISA test results are caused by the specific pathogenesis of ParaTB (Collins, 2003). The synthesis of antibodies is causally linked to the stage of the disease. The first step after the infection of immune cells by MAP is the cell-mediated immune response to MAP. The next step in the progress to Johne's disease is the change from the cell-mediate to the humoral immune response (Wu *et al*., 2007). Depending on time of testing and the used test to determine the MAP infection status it is obvious to get different test results within the

same animal, based on the different stages in the pathogenesis of ParaTB. In fecal shedders without clinical symptoms it is not possible to detect antibodies against MAP in contrast to animals with clinical disease (Collins *et al*., 1991; Sweeney *et al*., 1995; Dargatz *et al*., 2001). Therefore, the age of testing (Nielsen and Toft, 2006) and the level of shedding (Sweeney *et al*., 1995; Whitlock *et al*., 2000; Dargatz *et al*., 2001) influence the serological test result. A causal relationship between the increased antibody synthesis and enhanced MAP shedding is still not established, but a higher lysis of macrophages in later stages of the disease is assumed to cause a higher antigen presentation to T- and B-cells and with this an increase in antibody secretion (Coussens, 2001). The discussed complexity of the test methods definitely shows an influence on the results of the GWA studies. Depending on the different test methods or grouping of test methods we get different results and therefore different candidate genes associated with MAP infection. Furthermore, the problem of correct phenotyping discussed here leads to the conclusion that GWA-Meta-Analysis should only be done within the same test method.

Table 8: Results of the different test methods for MAP. Number of fecal culture positive and fecal culture negative animals for *Mycobacterium avium* ssp. *paratuberculosis* and the break down of the four different ELISA tests.

ELISA test		fecal POS	fecal NEG
		n=152	n=153
CATTLETYPE®	POS	87	17
	NEG	65	136
ID Screen®	POS	91	23
	NEG	61	130
Parachek®	POS	87	24
	NEG	65	129
Pourquier®	POS	91	21
	NEG	61	132

Conclusion for prospects concerning further studies in genetic of MAP-infection

One main point seems to be the improvement of diagnostic for MAP infection to get a correct and consistent phenotype for further studies concerning ParaTB. Further region-wide monitoring seems to be necessary to improve the power of further genetic studies resulting in verified statements. The number of tested animals, at best with whole pedigree information, is a limitation factor beside the phenotyping method for genetic studies. Both improvement of phenotyping and the increasing number of animals are also basic factors for

other approaches like study of epigenetic factors (e.g. within the *NOD2*), expression studies, or whole genome sequencing.

7. SUMMARY

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the causative agent for the global occurrence of paratuberculosis (Johne's disease) in ruminants. Infection with MAP leads to chronic enteritis and is responsible for economic losses, especially for dairy farmers. Because of cost and time efficiency the majority if studies about MAP infection deal with ELISA test results for phenotyping. The present study demonstrates with a dataset of 9 367 fecal culture tested German Holstein cows that the MAP status of the animals, the farm and the lactation number, as well as the interaction between MAP status and farm significantly affect the milk parameters, especially milk yield in kilograms. Also the interaction between lactation number and MAP status is significantly associated with milk kg per day of life. Although, if only a part of the real losses could be considered, the presented results of MAP infection to production traits underline the economic importance to control and eradicate the disease.

So far, there is no therapy or vaccination against MAP, but there are suggestions that susceptibility to MAP infection has a genetic component. To verify the hypothesis also for fecal culture tested animals, a large dataset of 11 285 fecal culture tested German Holstein cows were used to estimate heritability for MAP infection. With results between 0.157 and 0.228 it could be demonstrated that the fecal culture test method is more suitable for further studies than ELISA test results. Furthermore, the estimated heritabilities are comparable with other traits which are already included successfully in breeding programs.

Because of the genetic background of the disease, numerous studies deal with the analysis of possible candidate genes and their association with MAP infection as well as with genome-wide association (GWA) studies to identify genetic region of interests with MAP infection. Amongst others the *nucleotide-binding oligomerization domain containing 2* (*NOD2*, formerly *CARD15*) was described as a candidate gene for susceptibility with MAP infection in cattle. In the present study eleven SNPs in the *NOD2* gene could be identified, and finally, four SNPs were included in a case-control study using 324 German Holstein cows tested for paratuberculosis using fecal culture and ELISA. Thereby the SNP (GenBank) AY518738S04:g.521G>A in exon 4 showed a significant association (*P = 0.0056*) between the fecal culture status of the animals and *NOD2* allele variants. Furthermore a GWA study was performed with the Illumina Bovine SNP50BeadChip using a case-control assay of 305 cows

tested for MAP by fecal culture with an additional four different commercial ELISA-tests. No so far reported association for MAP infection could be confirmed. However, new regions of interest and potential candidate genes could be found. These results lead to the conclussion, that different test methods for MAP infection result in diverse identified gene loci, and therefore in different candidate genes associated with MAP. Furthermore it seems to be once more, that paratuberculosis is not affected by just one gene.

The results of the present study are reflecting the problem of the diagnostic of MAP infection. The different test methods and the different grouping of phenotyping leads to different results of the studies. Therefore, further improvement of diagnostic for MAP infection is essential to get a correct and consistent phenotype for further studies concerning MAP infection. Beside which, the phenotyping of the large number of tested animals, at best with whole pedigree information, is a limitation factor for genetic studies. Both improvement of phenotyping and the increasing number of animals are basic factors for further genetic studies of MAP infection.

8. ZUSAMMENFASSUNG

Mycobacterium avium ssp. *paratuberculosis* (MAP) ist die Ursache für die bei Wiederkäuern ubiquitär vorkommende Krankheit Paratuberkulose. Eine Infektion mit MAP führt bei den betroffenen Tieren zu einer chronischen Darmentzündung und hat damit eine entsprechende Leistungsminderung zur Folge. Mit MAP infizierte Tiere können daher die Ursache für geringere Leistungen in Milch produzierenden Betrieben und somit ursächlich für erhebliche ökonomische Verluste sein.

Im Rahmen der vorliegenden Arbeit konnte auf Daten von 9.367 Deutsche Holstein-Kühen zurückgegriffen werden. Diese Kühe wurden im Gegensatz zu zahlreichen anderen Studien mittels Kotkulturen auf MAP-Infektionen getestet. Diese Methode ist zwar nicht so zeit- und kosteneffizient wie die weithin genutzten ELISA-Tests, liefert aber verlässlichere Ergebnisse. Die Analysen dieser Daten innerhalb der vorliegenden Arbeit zeigten, dass sowohl der MAP-Status, der Betrieb und die Laktationsnummer als auch die Interaktion zwischen dem MAP-Status und dem Betrieb einen signifikanten Effekt auf verschiedene Milchparameter (insbesondere die Milchmenge) haben. Darüber hinaus ist die Interaktion zwischen MAP-Status und Laktationsnummer signifikant mit dem Merkmal „Milchkilogramm pro Lebenstag“ assoziiert. Die nachgewiesenen Assoziationen des MAP-Status mit der Milchleistung von Deutsche Holstein-Kühen und die damit verbundenen wirtschaftlichen Einbußen unterstreichen die Notwendigkeit, Paratuberkulose zu kontrollieren und zu bekämpfen.

Bisher gibt es keine erfolgreiche Therapie oder zugelassene Impfung gegen Paratuberkulose, jedoch wird eine genetische Komponente im Zusammenhang mit MAP vermutet, so dass längerfristig eine Zucht gegen Paratuberkulose möglich sein könnte. Um diese genetischen Einflüsse zu überprüfen, wurde innerhalb der vorliegenden Arbeit ein Datensatz von 11.285 mittels Kotkultur getesteten Tieren verwendet, um die Erblichkeit für MAP-Infektionen zu ermitteln. Mit Heritabilitäten zwischen 0,157 und 0,228, die im gleichen Bereich liegen wie Erblichkeiten anderer Merkmale, die bereits erfolgreich in Zuchtprogramme eingebunden sind, konnte gezeigt werden, dass die Kotkultur im Vergleich zu ELISA eine geeignetere Methode für zukünftige Studien ist.

Aufgrund des genetischen Hintergrunds der Krankheit wurden bereits zahlreiche Kandidatengenanalysen und genomweite Assoziationsstudien (GWA) zur Identifizierung

interessanter Genregionen im Zusammenhang mit MAP Infektionen durchgeführt. Darunter wurde das *nucleotide-binding oligomerization domain containing 2* (*NOD2*, früher *CARD15*) als Kandidatengen im Zusammenhang mit Paratuberkulose bei Rindern beschrieben. In der vorliegenden Studie wurden elf SNPs im *NOD2*-Gen identifiziert, von denen letztendlich vier in eine Fall-Kontroll-Studie mit 324 Deutsche Holstein-Kühen verwendet wurden. Die Tiere wurden mittels Kotkultur und ELISA auf MAP getestet. Dabei zeigte der SNP (GenBank) AY518738S04:g.521G>A in Exon 4 des *NOD2* eine signifikante Assoziation (*P = 0,0056*) zum Kotkulturstatus der Tiere. Des Weiteren wurde eine GWA-Studie mithilfe des Illumina Bovine SNP50 BeadChip durchgeführt. Hierfür wurde eine Fall-Kontroll-Studie mit 305 Kühen durchgeführt, welche zum einen mit Kotkultur und zum anderen mit vier kommerziellen ELISA-Tests auf MAP getestet wurden. Zwar konnte keine der bisher beschriebenen Assoziationen bestätigt werden, jedoch konnten weitere interessante Genregionen und potentielle Kandidatengene identifiziert werden. Zum einen konnte dadurch gezeigt werden, dass durch die unterschiedlichen Testmethoden für MAP verschiedene Genregionen identifiziert werden und damit unterschiedliche potentielle Kandidatengene für Paratuberkulose, und zum anderen, dass mehrere Genregionen beim Krankheitsgeschehen der Paratuberkulose beteiligt sind.

Die Ergebnisse der vorliegenden Studie reflektieren die Problematik der Diagnose für MAP-Infektionen: Die unterschiedlichen Testmethoden und die sich daraus ergebende unterschiedliche Gruppierung des Phänotyps führen zu unterschiedlichen Ergebnissen innerhalb der Studien. Neben der Phänotypisierung großer Tierzahlen, möglichst mit vollständigen Pedigree-Informationen, sind daher Verbesserungen der Diagnostik für MAP vonnöten, um einheitliche und wiederholbare Ergebnisse zur Phänotypisierung für weitere Studien zu erhalten. Beides - sowohl die Verbesserung der Phänotypisierung wie auch eine erhöhte Anzahl untersuchter Tiere - ist eine Grundvoraussetzung für informative genetische Untersuchungen im Zusammenhang mit MAP-Infektionen.

9. APPENDIX

Appendix Table 1: Samples of non-ruminants animals with MAP infection from different countries.

Species	Country	Author
Rabbits	Scotland	Greig *et al*., 1999; Judge *et al*., 2006
	USA	Raizman *et al.,* 2005
	Spain	Maio *et al*., 2011
Brown hare	Greek	Florou *et al*., 2008
Cottontail, armadillo	USA	Corn *et al*., 2005
Rats	Scotland	Beard *et al*., 2001
	USA	Corn *et al*., 2005
	Greek	Florou *et al*., 2008
	Czech Republic	Kopecna *et al*., 2008
Different mice	Scotland	Beard *et al*., 2001
	USA	Corn *et al*., 2005
	Greek	Florou *et al*., 2008
	Czech Republic	Kopecna *et al*., 2008
Stoats, weasels and badgers	Scotland	Beard *et al*., 2001
Wild boar	Spain	Álvarez *et al*., 2005
	Czech Republic	Kopecna *et al*., 2008
Foxes	Scotland	Beard *et al*., 2001
	USA	Anderson *et al*., 2007
	Greek	Florou *et al*., 2008
Raccoon, opossum, striped skunk	USA	Corn *et al*., 2005; Anderson *et al*., 2007
Feral cat	USA	Corn *et al*., 2005; Palmer *et al*., 2005; Anderson *et al*., 2007
Different kinds of birds	Scotland	Beard *et al.,* 2001
	USA	Corn *et al.,* 2005
	Slovakia	Gronesova *et al.,* 2008

Appendix Table 2: Prevalences in Europe for infection with *Mycobacterium avium* ssp. *paratuberculosis* in cattle.

Country /Region	Prevalence (%)	Level	Test	Age-group	N =	Reference
Austria	6.0	animal	ELISA[1]	Four oldest/herd	11 028	Gasteiner *et al*. (1999)
	19.0	animal	ELISA[2]	Culled cattle <2 years	756	Dreier *et al*. (2006)
	5.0	animal	fecal culture or qPCR[§]	> 1.5 years	510	Kohl *et al.* (2013)
	2.1 – 5.1	animal	ELISA[2] milk		439	
	14.9 – 18.8	animal	ELISA[2]			
Belgium	Ø 18.0 dairy: 32.0 beef: 7.0	herd	ELISA[3]	> 2 years	13 150	Boelaert *et al.* (2000)
	0.9	animal				
	Ø 2.2 dairy: 3.9 beef: 1.2 mixed: 2.7	animal	ELISA[3]	> 24 months	13 616	Vangeel *et al*. (2012)
	21.6	herd		> 24 months	937 herds	
Denmark	8.8	animal	ELISA[1] milk	n.s.[+]	1 155	Jakobsen *et al.* (2000)
	70.0	herd	ELISA[1] bulk-tank milk	n.s.	900 herds	Nielsen *et al*. (2000)
France	3.3	animal	ELISA[4]	All	8 793	Petit (2001)
	68.0	herd	ELISA[4]	All	8 793	
Netherlands	55.0	herd	ELISA[3]	≥ 3 years	15 822 378 herds	Muskens *et al*. (2000)
Republic of Ireland	3.7 (3.6)[*]	animal	ELISA[5]	n.s.	225	O'Doherty *et* al. (2002)
	25.0	herd	ELISA[5]		225	
	4.1	animal	Fecal culture		221	
	37.5	herd	Fecal culture		221	
	12.9	herd	IS*900* PCR[#], bulk-tank milk	n.s.	389 samples	O'Reilly *et al.* (2004)
	2.9 dairy: 2.7 beef: 3.1	animal	ELISA[4]	> 12 months	20 322	Good *et al.* (2009)
	21.4 dairy: 31.5 beef: 17.9	herd	ELISA[4]	> 12 months	639 herds	
Italy	64.9	herd	ELISA[3]	> 12 months	419 herds	Robbi *et al.* (2002)
	42.0	herd	ELISA[3]		369 herds	Lillini *et al*. (2005)
	2.4	animal	ELISA[3]		19 627	
Spain	8.0-10.0	herd	IS*900* PCR, bulk-tank milk	n.s.	200 samples	Sevilla *et al.* (2002)
Swizerland						
	19.7	herd	IS*900* PCR, bulk-tank milk	n.s.	1 384 samples	Corti and Stephan (2002)

Turkey	6.2	animal	ELISA[4]	> 2 years	465	Ozturk *et al*. (2010)
	58.3	herd	ELISA[4]	> 2 years	465	
	9.5-20.0	animal	Fecal bacterioscopy and fecal culture	3 -7 years	200	Yildirim and Civelek (2013)
	5.5-17.5	animal	Milk bacterioscopy and fecal culture	3 -7 years	200	
United Kingdom	2.3	animal	Self-administered questionnaire	n.s.	2 915 herds	Cetinkaya *et al*. (1998)
	17.4	herd	Self-administered questionnaire			

[1]Allied, [2]Svanovir, [3]IDEXX, [4]Pourquier, [5]Paracheck

[§]qPCR = real-time polymerase chain reaction, [#]PCR = polymerase chain reaction

[+]not specified

[*]recalculated

10. REFERENCES OF THE GENERAL PART

Àlvarez, L., de Juan, L., Briones, V., Romero, B., Aranaz, A., Fernández-Garayzábal, J.F. & Mateos, A. (2005). *Mycobacterium avium* subspecies *paratuberculosis* in fallow deer and wild boar in Spain. Vet. Rec. 156: 212-213.

Aly, S.S., Anderson, R.J. Adaska, J.M., Jiang, J. & Gardner, I.A. (2010). Association between *Mycobacterium avium* subspecies *paratuberculosis* infection and milk production in two California dairies. J. Dairy Sci. 93: 1030-1040.

Anderson, J.L., Meece, J.K., Koziczkowski, J.J., Clark Jr., D.L., Radcliff, R.P., Nolden, C.A., Samuel, M.D. & Ellingson, J.L.E. (2007). *Mycobacterium avium* subsp. *paratuberculosis* scavenging mammals in Wisconsin. J. Wildlife Dis. 43: 302-308.

Ansari-Lari, M., Haghkhah, M & Mahmoodi, F. (2012). Association of *Mycobacterium avium* subspecies *paratuberculosis* infection with milk production and calving interval in Iranian Holsteins. Trop. Anim. Health. Prod. 44: 1111-1116.

Attalla, S.A., Seykora, A.J., Cole, J.B. & Heins, B.J. (2010). Genetic parameters of milk ELISA scores. J. Dairy Sci. 93: 1729-1735.

Autschbach, F., Eisold, S., Hinz, U., Zinser, S., Linnebacher, M., Giese, T., Löffler, T., Büchler, M.W. & Schmidt, J. (2005). High prevalence of *Mycobacterium avium* subspecies *paratuberculosis* IS*900* DNA in gut tissue from individuals with Crohn's disease. Gut 54: 944-949.

Bang, B. (1906). Chronische pseudotuberkulöse Darmentzündung beim Rinde. Berl. Tierärztl. Wschr. 22: 759-763.

Beard, P.M., Daniels, M.J., Henderson, D., Pirie, A., Rudge, K., Buxton, D., Rhind, S., Greig, A., Hutchings, M.R., Mc Kendrick, I., Stevenson, K. & Sharp, J.M. (2001). Paratuberculosis infection of nonruminant wildlife in Scotland. J. Clin. Microbiol. 39: 1517-1521.

BGBl (2013). Verordnung über meldepflichtige Krankheiten in der Fassung der Bekanntmachung vom 11. Februar 2011 (BGBl. I S. 252), die zuletzt durch Artikel 2 der Verordnung vom 12. Juni 2013 (BGBl. I S. 1576) geändert worden ist. BGBl. I 29: S. 1576.

Bhattarai, B., Fosgate, G.T., Osterstock, J.B., Fossler, C.P., Park, S. & Roussel, A.J. (2013). Perceptions of veterinarians in bovine practice and producers with beef cow-calf operations enrolled in the US Voluntary Bovine Johne's Disease Control Program concerning economic losses associated with Johne's disease. Prev. Vet. Med. 112: 330-337.

Bisping, W. & Amtsberg G. (1988). Farbatlas zur Diagnose bakterieller Infektionserreger der Tiere. Verlag Parey, Berlin, Hamburg, p. 119-120.

BMVEL (2005). Leitlinien für den Umgang mit der Paratuberkulose in Wiederkäuerbeständen (Paratuberkuloseleitlinien). BAnz. 28: 2165-2166.

Boelaert, F., Walravens, K., Biront, P., Vermeersch, J.P., Berkvens, D. & Godfroid, J. (2000). Prevalence of paratuberculosis (Johne's disease) in the Belgian cattle population. Vet. Microbiol. 77, 269-281.

Böttcher, M. (1997). Kulturelle und serologische Untersuchungen zur Prävalenz der Paratuberkulose im Regierungsbezirk Arnsberg. Doctoral thesis, Tierärztliche Hochschule Hannover, Germany, p. 74.

Böttcher, J. & Gangl, A. (2004). *Mycobacterium avium* ssp. *paratuberculosis* – combined serological testing and classification of individual animals and herds. J. Vet. Med. B 51, 443-448.

Bull, T.J., McMinn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R. & Hermon-Taylor, J. (2003). Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in Fresh Ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. J. Clin. Microbiol. 41: 2915-2923.

Carta, T. Álvarez, J., Pérez de la Lastra, J.M. & Gortázar (2013). Wildlife and paratuberculosis: A review. Res. Vet. Sci. 94: 191-197.

Çetinkaya, B., Erdogan, H.M. & Morgan, K.L. (1998). Prevalence, incidence and geographical distribution of Johne's disease in cattle in England and the Welsh borders. Vet. Rec. 143: 265-269.

Chacon, O., Bermudez, L.E. & Barletta, R.G. (2004). Johne's disease, inflammatory bowel disease, and *Mycobacterium paratuberculosis.* Annu. Rev. Microbiol. 58: 329-363.

Chi, J., VanLeeuwen J.A., Weersink, A. & Keefe, G.P. (2002). Direct production losses and treatment costs from bovine viral diarrhea virus, bovine leucosis virus, *Mycobacterium avium* subspecies *paratuberculosis*, and Neospora caninum. Prev. Vet. Med. 55: 137-153.

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.

Chiodini , R.J. (1989). Crohn's disease and the Mycobacterioses: a review and comparison of two disease entities. Clinic. Microbiol. Rev. 2: 90-117.

Clarke, C.J. (1997). Review: The Pathology and Pathogenesis of Paratuberculosis in Ruminants and Other Species. J. Comp. Path. 116: 217-261.

Cocito, C., Gilot, P., Coene, M., De Kesel, M., Poupart, P. & Vannuffel, P. (1994). Paratuberculosis. Clin. Microbiol. Rev. 7: 328-345.

Collins, D.M., Gabric, D.M. & De Lisle, G.W. (1989). Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. FEMS Microbiol. Lett. 60: 175-178.

Collins, M.T., Sockett, D.C., Ridge, S. & Cox, J.C. (1991). Evaluation of a commercial enzyme-linked Immunosorbent assay for Johne`s disease. J. Clin. Microbiol. 29: 272-276.

Collins, M.T. (2003). Paratuberculosis: Review of present knowledge. Acta. Vet. Scand. 44: 217-221.

Collins, M.T., Wells, S.J., Petrini, K.R., Collins, J.E., Schultz, R.D. & Whitlock, R.H. (2005). Evaluation of Five Antibody Detection Tests for Diagnosis of Bovine Paratuberculosis. 12: 685-692.

Collins, M.T., Eggleston, V. & Manning, E.J.B. (2010). Successful control of Johne's disease in nine dairy herds: Results of a six-year field trial. J. Dairy Sci. 93: 1638-1643.

Corn, J.L., Manning, E.J.B., Sreevatsan, S. & Fischer, J.R. (2005). Isolation of *Mycobacterium avium* subsp. *paraubercul*osis from free-ranging birds and mammals on livestock Premises. Appl. Environ. Microbiol. 71: 6963-6967.

Corti, S. & Stephan, R. (2002). Detection of *Mycobacterium avium subspecies paratuberculosis* specific IS900 insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. BMC Microbiol. 2: 15.

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

Dalziel, T.K. (1913). Chronic intestinal enteritis. BMJ 2: 1068-1070.

Dargatz, D.A., Byrum, B.A., Barber, L.K., Sweeney, R.W., Whitlock, R.H., Shulaw, W.P., Jacobson, R.H. & Stabel, J.R. (2001). Evaluation of a commercial ELISA for diagnosis of paratuberculosis in cattle. J. Am. Vet. Med. Assoc. 218: 1163-1166.

Davis, G., Genini, S., Bishop, S.C. & Giuffra, E. (2009). An assessment of opportunities to dissect host genetic variation in resistance to infectious diseases in livestock. Animal 3: 415-436.

Donat, K., Eulenberger, K. & Kampfer, P. (2005). Blutserologische Untersuchungen zur Verbreitung von *Mycobacterium avium* spp. *Paratuberculosis* in sächsischen Rinderbestanden. Tieraerztl. Umsch. 60, 497-501.

Donat, K. (2009). Umsetzung des Thüringer Paratuberkulose-Landesprogrammes auf der Basis des Erregernachweises - Ein Zwischenbericht. 7. Stendaler Symposium. Landesamt fur Verbraucherschutz Sachsen-Anhalt, Fachbereich 4 Veterinärmedizin, Stendal, Germany, p. 94.

Donat, K., Schau, U. & Soschinka, A. (2011). Identification of *Mycobacterium avium* ssp. *paratuberculosis* infected dairy herds by environmental sampling. Berl. Münch. Tierärztl. Wochenschr. 124: 360-367.

Donat, K., Schau, U., Soschinka, A. & Köhler, H. (2012). Herd prevalence studies of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in cattle using serological tests: opportunities, limitations and costs. Berl. Münch. Tierärztl. Wochenschr. 125: 360-367.

Donat, K., Soschinka, A., Erhardt, G. & Brandt, H. (2014). Paratuberculosis: decrease in milk production of German Holstein dairy cows shedding *Mycobacterium avium* ssp. *paratuberculosis* depends on within-herd prevalence. Animal doi:10.1017/S1751731114000305.

Doré, E., Paré, J., Côté, G., Buczinski, S., Labrecque, O., Roy, J.P. & Fecteau, G. (2012). Risk factors associated with transmission of Mycobacterium avium subsp. paratuberculosis to calves within dairy herd: a systematic review. J. Vet. Intern. Med. 26: 32-45.

Douarre , P.E., Cashman, W., Buckley, J., Coffey, A. & O'Mahoni, J.M. (2010). Isolation and detectin of Mycobacterium avium subsp. Paratuberculosis (MAP) from cattle in Ireland using both traditional culture and molecular based methods. Gut Pathog. 2: 11.

Dreier, S., Khol, J.L., Stein, B., Fuchs, K., Gutler, S. & Baumgartner, W. (2006). Serological, bacteriological and molecularbiological survey of paratuberculosis (Johne's disease) in Austrian cattle. J. Vet. Med. B 53: 477-481.

Fan, B., Du, Z.-Q., Gorbach, D.M. & Rothschild, M.F. (2010). Development and Application of High-density SNP Arrays in Genomic Studies of Domestic Animals. Asian-Aust. J. Anim. Sci. 23: 833-847.

Fecteau M.-E., Whitlock, R.H., Buergelt, C.D. & Sweeney, R.W. (2010). Brief communication: Exposure of young dairy cattle to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) through intensive grazing of contaminated pastures in a herd positive for Johne's disease. Can. Vet. J. 51: 198-200.

Felaco, P., Castellani, M.L., de Lutiis, M.A., Felaco, M., Pandolfi, F., Salini, V., de Amicis, D., Vecchiet, J., Tete, S., Ciampoli, C., Conti, F., Cerulli, G., Caraffa, A., Antinolfi, P., Cuccurullo, C., Perrella, A., Theoharides, T.C., Conti, P., Toniato, E., Kempuraj, D. & Shaik, Y.B. (2009). IL-31: a newly-discovered proinflammatory cytokine. J. Biol. Regul. Homeost. Agents. 23: 141-147.

Florou, M., Leontides, L., Kostoulas, P., Billinis, C., Sofia, M., Kyriazakis, I. & Lykotrafitis, F. (2008). Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from non-ruminant wildlife in the sheds and on the pastures of Greek sheep and goat. Epidemiol. Infect. 136: 644-652.

Fujino, S., Andoh, A., Bamba, S., Ogawa, A., Hata, K., Araki, Y., Bamba, T. & Fujiyama, Y. (2003). Increased expression of interleukin 17 in inflammatory bowel disease. Gut 52: 65-70.

Gabriel S.B., Schaffner S.F., Nguyen H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, M., Faggart, M., Liu-Cordero, S.N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E.S., Daly, M.J. & Altshuler, D. (2002). The Structure of Haplotype Blocks in the Human Genome. Science 296: 2225-2229.

Gasteiner, J., Wenzl, H., Fuchs, K., Jark, U. & Baumgartner, W. (1999). Serological cross-sectional study of paratuberculosis in cattle in Austria. J. Vet. Med. B 46, 457-466.

Gasteiner, J., Awad-Masalmeh, M. & Baumgartner, W. (2000). Mycobacterium avium subsp. Paratuberculosis infection in cattle in Austria, diagnosis with culture, PCR and ELISA. Vet. Microbiol 77: 339-349.

GenBank (2013). http://www.ncbi.nlm.nih.gov/genbank/. Download: 06.12.2013.

Gerlach, G.-F. (2002). Paratueculosis: the pathogenen and routes of infection. Dtsch. Tierärztl. Wschr. 109: 504-506.

Gerold, G., Zychlinsky, A. & deDiego J. L. (2007). Review: What is the role of Toll-like receptors in bacterial infections? Semin. Immunol. 19: 41-47.

Gilardoni, L.R., Paolicchi, F.A. & Mundo S.L. (2012). Bovine paratuberculosis: a review of the advantages and disadvantages of different diagnostic tests. Rev. Argent. Microbiol. 44: 201-215.

Goddard, M.E., Hayes, B.J. & Meuwissen, T.H.E. (2010). Genomic selection in livestock populations. Genet. Res., Camb. 92: 413-421.

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.

Gonda, M.G., Kirkpatrick, B.W., Shook, G.E. & Collins M.T. (2007). Identification of a QTL on BTA20 affecting susceptibility to *Mycobacterium avium* ssp. *paratuberculosis* infection in US Holsteins. Anim. Genet. 38: 389-396.

Good, M., Clegg, T., Sheridan, H., Yeasely, D., O'Brian T., Egan, T. & Mullowney, P. (2009). Prevalence and distribution of paratuberculosis (Johne's disease) in cattle herds in Ireland. Irish Vet. J. 62: 597-606.

Greig, A., Stevenson, K., Henderson, D., Perez, V., Hughes, V., Pavlik, I., Hines II, M.E., Mc Kendrick, I. & Sharp, J.M. (1999). Epidemiological study of paratuberculosis in wild rabbits in Scotland. J. Clin Microbiol. 37: 1746-1751.

Gronesova, P., Ficova, M., Mizakova, A., Kabat, P., Trnka, A. & Betakova, T. (2008). Prevalence of avian influenza viruses, Borrelia garinii, Mycobacterium avium, Mycobacterium avium subsp. paratuberculosis in waterfowland terrestrialbirds in Slovakia, 2006. Avian Pathol. 37: 537-543.

Hacker, U., Hüttner, K. & Konow, M. (2004). Untersuchungen zur serologischen Prävalenz und zu Risiskofaktoren der Paratuberkulose in Milchviehbetrieben in Mecklenburg-Vorpommern. Berl. Münch. Tierärztl. Wschr. 117: 140-144.

Harris, N. B. & Barletta, R.G. (2001). *Mycobacterium avium* ssp. *paratuberculosis* in veterinary medicine. Clin. Microbiol. Rev. 14: 489-512.

Hendrick, S.H., Kelton, D.F., Leslie, K.E., Lissemore, K.D., Archambault, M. & Duffield, T.F. (2005a). Effect of paratuberculosis on culling, milk production, and milk quality in dairy herds. J. Am. Vet. Med. Assoc. 227: 1302-1308.

Hendrick, S., Duffield, T., Leslie, K., Lissemore, K., Archambault, M. & Kelton, D. (2005b). The prevalence of milk and serum antibodies to *mycobacterium avium* subspecies *paratuberculosis* in dairy herds in Ontario. Can. Vet. J. 46: 1126-1129.

Hinger, M., Brandt, H. & Erhardt, G. (2008). Heritability estimates for antibody response to *Mycobacterium avium* subspecies *paratuberculosis* in German Holstein cattle. J. Dairy Sci. 91: 3237-3244.

Hinger, M. (2009). Untersuchungen genetischer Ursachen der unterschiedlichen Empfänglichkeit für die Paratuberkulose des Rindes anhand von Mikrosatelliten- und Kandidatengenanalyse. Doctoral thesis, Justus-Liebig-University Gießen. VVB Laufersweiler Verlag.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cézard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.-F., Sahbatou, M. & Thomas, G. (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411: 599-603.

Jakobsen, M.B., Alban, L. & Nielsen, S.S. (2000). A cross-sectional study of paratuberculosis in 1155 Danish dairy cows. Prev. Vet. Med. 46: 15-27.

Johne, H.A. & Frothingham, L. (1895). Ein eigentümlicher Fall von Tuberkulose beim Rind. Deutsch. Z. Thiermed. Path. 21: 438-454.

Johnson-Ifearulundu, Y. & Kaneene, J.B. (1998). Management-related risk factors for *M. paratuberculosis* infection in Michigan, USA, dairy herds. Prev. Vet. Med. 37: 41-54.

Johnson-Ifearulundu, H.J., Kaneene, J.B., Sprecher, D.J., Gardiner, J.C. & Lloyd, J.W. (2000). The effect of subclinical Mycobacterium paratuberculosis infection on days open in Michigan, USA, dairy cows. Prev. Vet. Med. 46: 171-181.

Judge, J., Kyriazakis, I., Greig, A., Davidson, R.S. & Hutchings, M.R. (2006). Routes of Intraspecies Transmission of *Mycobacterium avium* subsp. *paratuberculosis* in rabbits (*Oryctolagus cuniculus*): a field study. Appl. Environ. Microbiol. 72: 398-403.

Khol, J.L., Damoser, J., Dünser, M. & Baumgartner, W. (2007). Paratuberculosis, a notifiable disease in Austria – current status, compilsory measures and first experiences. Prev. Vet. Med. 82: 302-307.

Khol, J.L. & Baumgartner, W. (2012). Examples and Suggestions for the Control of Paratuberculosis in European Cattle. Jpn. J. Vet Res. 60: S1-S7.

Kohl, J.L., Wassertheuer, M., Sodoma, E., Revilla-Fernández, S., Damoser, J., Österreicher, E., Dünser, M., Kleb, U. & Baumgartner, W. (2013). Long-term detection of *Mycobacterium avium* subspecies *paratuberculosis* in individual and bulk tank milk from a dairy herd with low prevalence of Johne's disease. J. Dairy Sci. 96: 3517-3524.

Kirkpatrick, B.W., Shi, X., Shook, G.E. & Collins, M.T. (2011). Whole-genome association analysis of susceptibility to paratuberculosis in Holstein cattle. Anim. Genet. 42: 149-160.

Köhler, H., Gyra, H., Zimmer, K., Dräger, K.G., Burkert, B., Lemser, B., Hausleithner, D., Cußler, K., Klawonn, W. & Heß, R.G. (2001). Immune Reaction in Cattle after Immunization with a Mycobacterium paratuberculosis Vaccine and Implications for the Diagnosis of *M. paratuberculosis* and *M. bovis* infection. J. Vet. Med. B 48: 185-195.

Köhler, H., Burkert, B., Pavlik, I., Diller, R., Geue, L., Conraths, F.J. & Martin, G. (2008). Evaluation of five ELISA test kits for the measurement of antibodies against Mycobacterium avium subspecies paratuberculosis in bovine serum. Berl. Münch. Tierärztl. Wochenschr. 121: 203-210.

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

Kopecna, M., Trcka, I., Lamka, J., Moravkova, M., Koubek, P., Heroldova, M., Mrlik, V., Kralova, A. & Pavlik, I. (2008). The wildlife hosts of *Mycobacterium avium* subsp. *paratuberculosis* in the Czech Republic during the year 2002 – 2007. Vet. Med. 53: 420-426.

Küpper, J., Chessa, S., Rignanese, D., Caroli, A. & Erhardt, G. (2010). Divergence at the casein haplotypes in dairy and meat goat breeds. J. Dairy Res. 77: 56-62.

Lillini, E., Bitoni, G., Gamberale, F. & Cersini, A. (2005). Prevalence of bovine paratuberculosis in the Latium region (Italy). Proceedings of the 8th Internaional Colloquium on Paratuberculosis, 14. - 18.08.2005, Copenhagen, Denmark, p. 638-644.

Lombard, J.E., Garry, F.B., McCluskey, B.J. & Wagner, B.A. (2005). Risk of removal and effects on milk production associated with paratuberculosis status in dairy cows. J. Am. Vet. Med. Assoc. 227: 1975-1981.

Lombard, J.E., Gardner, I.A., Jafarzadeh, S.R., Fossler, C.P., Harris, B., Capsel, Wagner, B.A. & Johnson, W.O. (2013). Herd-level prevalence of Mycobacterium avium subsp. paratuberculosis infection in United States dairy herds in 2007. Prev. Vet. Med. 108: 234-238.

Mackintosh, C.G. & Griffin, J.F. (2010). *Mycobacterium* avium Complex. In: Paratuberculosis Organism, Disease, Control, Eds: Behr, M.A. & Collins, D.M., Wallingford: CAB International: 179-187.

Maio, E., Carta, T., Balseiro, A., Sevilla, I.A., Romano, A., Ortiz, J.A., Vieira-Pinto, M., Garrido, J.M., de la Lastra, J.M.P. & Gortázar, C. (2011). Paratuberculosis in European rabbits from the Iberian Peninsula. Res. Vet. Sci. 91: 212-218.

McKenna, S.L.B., Keefe, G.P., Barkema, H.W. & Sockett, D.C. (2005a). Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. Vet. Microbiol. 110: 105-111.

McKenna, S.L.B., Sockett, D.C., Keefe, G.P., McClure, J., VanLeeuwen, J.A. & Barkema, H.W. (2005b). Comparison of two enzyme-linked immunosorbent assays for diagnosis of *Mycobacterium avium* subsp. *paratuberculosis*. J. Vet. Diagn. Invest. 17: 463-466.

McKenna, S.L.B., Keefe, G.P., Tiwari, A., VanLeeuwen, J.A. & Barkem, H.W. (2006). Review Article: Johne's disease in Canada. Part II: Disease impacts, risk factors, and control programs for dairy producers. Can. Vet. J. 47: 1089-1099.

Minozzi, G., Buggiotti, L., Stella, A., Strozzi, F., Luini, M. & Williams, J.L. (2010). Genetic loci involved in antibody response to *Mycobacterium avium* ssp. *paratuberculosis* in cattle. PLoS ONE 2010, 5:e11117.

Minozzi, G., Williams, J.L., Stella, A., Strozzi, F., Luini, M., Settles, M.L., Taylor, J.F., Whitlock, R.H., Zanella, R. & Neibergs, H.L. (2012). Meta-analysis of two genome-wide association studies of bovine paratuberculosis. PLoS ONE, 3:e32578.

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

Mucha, R., Bhide, M.R., Chakurkar, E.B., Novak, M. & Mikula, I. (2009). Toll-like receptors TLR1, TLR2 and TLR4 gene mutations and natural resistance to *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle. Vet. Immunol. Immunopathol. 128: 381-388.

Muskens, J., Barkema, H.W., Russchen, E., van Maanen, K., Schukken, Y.H. & Bakker, D. (2000). Prevalence and regional distribution of paratuberculosis in dairy herds in the Netherlands. Vet. Microbiol. 77: 253-261.

Muskens, J., van Zijderveld, F., Eger, A. & Bakker, D. (2002). Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. Vet. Microbiol. 86: 269-278.

Naser, S.A., Schwartz, D. & Shafran, I. (2000). Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from breast milk of Crohn's disease patients. Am. J. Gastroenterol. 95: 1094-1095.

Naser, S.A., Ghobrial, G., Romero, C. & Valentine J.F. (2004). Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's diseasae. Lancet 364: 1039-1044.

Nielsen, S.S., Thamsborg, S.M., Houe, H. & Bitsch, V. (2000). Bulk-tank milk ELISA antibodies for estimating the prevalence of paratuberculosis in Danish dairy herds. Prev. Vet. Med. 44: 1-7.

Nielsen, S.S., Grønbæk, C., Agger, J.F. & Houe, H. (2002a). Maximum-likelihood estimation of sensitivity and specificity of ELISAs and faecal culture for diagnosis of paratuberculosis. Prev. Vet. Med. 53: 191-204.

Nielsen, S.S., Enevoldsen, C. & Gröhn, Y.T. (2002b). The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. Prev. Vet. Med. 54: 1-10.

Nielsen, S.S. & Toft, N. (2006). Age-specific characteristics of ELISA and fecal culture for purpose-specific testing for paratuberculosis. J. Dairy Sci. 89: 569-579.

Nielsen, S.S. & Toft, N. (2008). Ante mortem diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon-γ assay and faecal culture techniques. Vet. Microbiol. 129: 217-235.

Nielsen, S.S. & Toft, N. (2009). Review: A review of prevalences of paratuberculosis in farmed animals in Europe. Prev. Vet. Med. 88: 1-14.

Nordlund, K.V., Goodger, W., Pelletier, J. & Collins, M. (1996). Association between subclinical paratuberculosis and milk production, milk components, and somatic cell counts in dairy herds. J. Am. Vet. Med. Assoc. 208: 1872-1876.

O'Doherty, A., O'Grady, D., O'Faell, K., Smith, T. & Egan, J. (2002). Short communication: Survey of Johne's disease in imported animals in the Republic of Ireland. Vet. Rec. 150: 634-636.

Oh, H.-M., Lee, H.-J., Seo, G.-E., Choi, E.-Y., Kweon, S.-H., Chun, C.-H., Han, W.-C., Lee, K.-M., Lee, M.-S., Choi, S.-C. & Jun, C.-D. (2005). Induction and localization of NOD2 protein in human endothelial cells. Cell. Immunol. 237: 37-44.

Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.A., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S.,

Hanauer, S.B., Nunez, G. & Cho, J.H. (2001). A frame shift mutation in NOD2 associated with Susceptibility to Crohn's disease. Nature 411: 603-606.

Okuni, J.B., Loukopoulos, P., Reinacher, M. & Ojok, L. (2011). Seroprevalence of Mycobacterium avium Subspecies Paratuberculosis antibodies in Cattle from Wakiso, Mpigi and Luwero Districts in Uganda. Int. J. Anim. Vet. Adv. 3: 156-160.

Okuni, J.B., Reinacher, M., Loukopoulos, P. & Ojok, L. (2013). Prevalence and spectrum of Johne's disease lesions in cattle slaughtered at two abattoirs in Kampala, Uganda. Trop. Anim. Health Prod. 45: 1197-1202.

O'Reilly, C.E., O'Connor, L., Anderson, W., Harvey, P., Grant, I.R., Donaghy, J., Rowe, M. & O'Mahony, P. (2004). Surveillance of Bulk Raw and Commercially Pasteurized Cows' Milk from Approved Irish Liquid-Milk Pasteurization Plants To Determine the Incidence of *Mycobacterium paratuberculosis*. Appl. Environ. Microbiol. 70: 5138-5144.

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.

Ozturk, D., Pehlivanoglu, F., Tok, A.A., Gunlu, S., Guldali, Y. & Turutoglu, H. (2010). Seroprevalence of paratuberculosis in the Burdur province (Turkey), in dairy cattle using enzyme linked immunosorbent assay (ELISA). Isr. J. Vet. Med. 65: 53-65.

Palmer, M.V., Stoffregen, W.C., Carpenter, J.G. & Stabel, J.R. (2005). Isolation of *Mycobacterium avium* subsp. *paratuberculosis* (Map) from Feral Cats on a Dairy Farm With Map-infected Cattle. J. Wildlife Dis. 41: 629-635.

Pant, S.D., Schenkel, F.S., Verschoor, C.P., You, Q., Kelton, D.F., Moore, S.S. & Karrow, N.A. (2010). A principal component regression based genome wide analysis approach reveals the presence of a novel QTL on BTA7 for MAP resistance in Holstein cattle. Genomics 95: 176-182.

Pappu, R., Ramirez-Carrozzi, V., Ota, N., Ouyang, W. & Hu, Y. (2010). The IL-17 family cytokines in immunity and disease. J. Clin. Immunol. 30: 185-195.

Paul-Ehrlich-Institut (2013). Rinderimpfstoffe, last update: BAnzAT 11.06.2013 B8. http://www.pei.de/DE/arzneimittel/impfstoff-impfstoffe-fuer-tiere/rinder/rinder-node.html. Download: 27.06.2013.

Petit, E. (2001). Enquete serologique sur la paratuberculose bovine menee dans l'Yonne lors de la campagne 98-99 (in French, with English abstract). Epidemiol. Sante Anim. 40: 23–39.

Pinedo, P.J., Buergelt, C.D., Donovan, G.A., Melendez, P., Morel, L., Wu, R., Langaee, T.Y. & Rae, D.O. (2009a). Candidate gene polymorphisms (BoIFNG, TLR4, SLC11A1) as risk factors for paratuberculosis infection in cattle. Prev. Vet. Med. 91: 189-196.

Pinedo, P.J., Wang, C. Li, Y., Rae, D.O. & Wu, R. (2009b). Risk haplotype analysis for bovine paratuberculosis. Mamm. Genome 20: 124-129.

Pinedo, P.J., Buergelt, C.D., Donovan, G.A., Melendez, P., Morel, L., Wu, R., Langaee, T.Y. & Rae, D.O. (2009c). Association between *CARD15*/*NOD2* gene polymorphisms and paratuberculosis infection in cattle. Vet. Microbiol. 134: 346-352.

Pritchard, T., Coffey, M., Mode, R. & Wall, E. (2013). Genetic parameters for production, health, fertility and longevity traits in dairy cows. Animal 7:34-36.

Purdie, A.C., Plain, K.M., Begg, D.J., de Silva, K. & Whittington, R.J. (2011). Candidate gene and genome-wide association studies of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and sheep: A review. Comp. Immunol. Microbiol. Infect. Dis. 34: 197-208.

Raizman, E.A., Wells, S.J., Godden, S.M., Bey, R.F., Oakes, M.J., Bentley, D.C. & Olsen, K.E. (2004). The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. J. Dairy Sci. 87: 2959-2966.

Raizman, E.A., Wells, S.J., Jordan, P.A., Del Giudice, G.D. & Bey, R.R. (2005). *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging deer and rabbits surrounding Minnesota dairy herds. Can. J. Vet. Res. 69: 32-38.

Raizman, E.A., Fetrow, J. & Wells, S. (2009). Loss of income from cows shedding *Mycobacterium avium* subspecies *paratuberculosis* prior to calving compared with cows not shedding the organism on two Minnesota dairy farms. J. Dairy Sci. 92: 4929-4936.

Ramirez-Valverde, R., Misztal, I. & Bertrand, J.K. (2001). Comparison of the threshold vs. linear and animal vs. sire models for predicting direct and maternal genetic effects on calving difficulty in beef cattle. J. Anim. Sci. 79: 333-338.

Richardson, E.K.B. & More, S.J. (2009). Direct and indirect effects of Johne's disease on farm and animal productivity in an Irish dairy herd. Ir. Vet. J. 62: 526-532.

Robbi, C., Rossi, I., Nardelli, S., Marangon, S., Vincenzi, G. & Vincenzoni, G. (2002). Prevalence of paratuberculosis (Johne's disease) in dairy farms in northeastern Italy. Proceedings of the 7th International Colloquium on Paratuberculosis, 11.-14.06.2002, Bilbao, Spain, p. 457-461.

Robinson, M.W., O'Brien, R., Mackintosh, C.G., Clark, R.G. & Griffin, J.F.T. (2011). Immunoregulatorycytokines are associated with protection from immunopathology following *Mycobacterium avium* subspecies *paratuberculosis* infection in red deer. Infect. Immun. 79: 2089-2097.

Romero C., Hamdi, A., Valentine, J.F. & Naser, S.A. (2005). Evaluation of surgical tissue from patients with Crohn's disease for the presence of *Mycobacterium avium* subspecies *paratuberculosis* DNA by in situ hybridization and nested polymerase chain reaction. Inflamm. Bowel Dis. 11: 116-125.

Rossiter, C. A. & Burhans, W.S. (1996). Farm-specific approach to paratuberculosis (Johnes-disease) control. Vet. Clin. North Am. - Food Anim. Practice 12: 383-415.

Rothschild, M.F., Hu, Z-I. & Jiang, Z. (2007). Advances in QTL mapping in pigs. Int. J. Biol. Sci. 3: 192-197.

Roussel, A.J., Libal, M.C., Whitlock, R.L., Hairgrove, T.B. Barling, K.S. & Thompson, J.A. (2005). Prevalence of and risk factors for paratuberculosis in purebred beef cattle. J. Am. Vet. Med. Assoc. 226: 773-778.

Rowbotham, D.S., Mapstone, N.P., Trejdosiewicz, L.K., Howdle, P.D. & Quirke, P. (1995). *Mycobacterium paratuberculosis* DNA not detected in Crohn's disease tissue by fluorescent polymerase chain reaction. Gut 33: 1445-147.

Ruiz-Larrañaga, O., Garrido, J.M., Manzano, C., Iriondo, M., Molina, E., Gils, A., Koets, A.P., Rutten, V.P.M.G., Juste, R.A. & Estonba, A. (2010a). Identification of single polymorphisms in the bovine solute carrier family 11 member 1 (*SLC11A1*) gene and their association with infection by *Mycobacterium avium* subspecies *paratuberculosis*. J. Dairy Sci. 93: 1713-1721.

Ruiz-Larrañaga, O., Garrido, J.M., Iriondo, M., Manzano, C., Molina, E., Koets, A.P., Rutten, V.P.M.G., Juste, R.A. & Estonba, A. (2010b). Genetic association between bovine *NOD2* polymorphisms and infection by *Mycobacterium avium* subsp. *paratuberculosis* in Holstein-Friesian cattle. Anim. Genet. 41: 652-655.

Sechi, L.A., Scanu, A.M., Molicotti, P., Cannas, S., Mura, M., Dettoti, G., Fadda, G. & Zanetti, S. (2005). Detection and isolation of *Mycobacterium avium* subspecies *paratuberculosis* from intestinal mucosal biopsies of patients with and withou Crohn's disease in Sardinia. Am. J. Gastroenterol. 100: 1529-1536.

Settles, M., Zanella, R., McKay, S.D., Schnabel, R.D., Taylor, J.F., Whitlock, R., Schukken, Y., van Kessel, J.S., Smith, J.M. & Neibergs, H. (2009). A whole genome association analysis identifies loci associated with *Mycobacterium avium* ssp. *paratuberculosis* infection status in US Holstein cattle. Anim. Genet. 40: 655-662.

Sevilla, I., Aduriz, G., Garrido, J.M., Geijo, M.V. & Juste, R.A. (2002). A preliminary survey on the prevalence of paratuberculosis in dairy cattle in Spain by bulk milk PCR. Proceedings of the 7th International Colloquium on Paratuberculosis, 11.-14.06.2002, Bilbao, Spain, p. 332-336.

Shu, D., Subharat, S., Wedlock, D.N., Luo, D., de Lisle, G.W. & Buddle, B.M. (2011). Diverse cytokine profile from mesenteric lymph nodl cells of cull cows severely affected with Johne's Disease. Clin. Vaccine Immuno. 18: 1467-1476.

Sorge, U.S., Kelon, D., Lissemore, K., Godkin, A., Hendrick, S. & Wells, S. (2010). Attitudes of Canadian dairy farmers towards a voluntary Johne's disease control program. J. Dairy Sci. 93: 1491-1499.

Stabel, J.R. (1996). Production of γ-interferon by peripheral blood mononuclear cells: an important diagnostic tool for detection of subclinical paratuberculosis. J. Vet. Diagn. Invest. 8: 345-350.

Statista (2013). http://de.statista.com/statistik/daten/studie/28934/umfrage/rinderbestand-in-deutschland-und-der-eu-seit-1990. Download: 22.04.2013.

Sweeney, R.W., Whitlock, R.H & Rosenberg, A.E. (1992a). *Mycobacterium paratuberculosis* Cultured from Milk and Supramammary Lymph Nodes of Infected Asymptomatic Cows. J Clin. Microbiol. 30: 166-171.

Sweeney, R.W., Whitlock, R.H & Rosenberg, A.E. (1992b). *Mycobacterium paratuberculosis* isolated from the fetuses of infected cows not manifesting signs of the disease. Am. J. Vet. Res. 4: 477-480.

Sweeney, R.W., Whitlock, R.H., Hamir, A.N., Rosenberg, A.E. & Herr, S.A. (1992c). Isolation of *Mycobacterium paratuberculosis* after oral inoculation of uninfected cattle. Am. J. Vet. Res. 53: 1312-1314.

Sweeney, R.W., Whitlock, R.H., Buckley, C.L. & Spencer, P.A. (1995). Evaluation of a commercial enzyme-linked immunosorbent assay for the diagnosis of paratuberculosis in dairy cattle. J. Vet. Diagn. Invest. 7: 488-493.

Sweeney, R.W. (1996). Transmission of paratuberculosis. Vet. Clin. North Am. Food 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., Collins, M.T., Koets, A.P., McGuirk, S.M. & Roussel, A.J. (2012). Paratuberculosis (Johne's Disease) in cattle and other susceptible species. J. Vet. Intern. Med. 26: 1239-1250.

Takeda, K., Kaisho, T., & Akira, S. (2003). Toll-like receptors. Annu. Rev. Immunol. 21: 335-376.

Taylor, K.H., Taylor, J.F., White, S.N. & Womack J.E. (2006). Identification of genetic variation and putative regulatory regions in bovine *CARD15*. Mam. Genome 17: 892-901.

Thomsen, V.T., Nielsen, S.S., Thakur, A. & Jungersen, G. (2012). Characterization of the long-term immune response to vaccination against *Mycobacterium avium* subsp. *paratuberculosis* in Danish dairy cows. Vet. Immunol. Immunopathol. 145: 316-322.

Timms, V.J., Gehringer, M.M., Mitchell, H.M. Daskalopoulos, G. & Neilan, B.A. (2011). Review: How accurately can we detect *Mycobacterium avium* subsp. *paratuberculosis* infection? J. Microbiol. Methods 85: 1-8.

Tiwari, A., Van Leeuwen, J.A., Dohoo, I.R. Keefe, G.P. Haddad, J.P., Tremblay, R., Scott, H.M. & Whitings, T. (2007). Production effects of pathogens causing bovine leukosis, bovine viral diarrhea, paratuberculosis, and neosporosis. J. Dairy Sci. 90: 659-669.

Urioste, J.I., Franzén, J., Windig, J.J. & Strandberg, E. (2012). Genetic relationships among mastitis and alternative somatic cell count traits in the first 3 lactations of Swedish Holsteins. J. Dairy Sci. 95: 3428-3434.

Vangeel, I., Méroc, E., Roelandt, S., Welby, S., Driessche, E.V., Czaplicki, G., Schoubroeck, L.V., Quinet, C., Riocreux, F., Hooyberghs, J., Houdart, P. & Stede, Y.V.d. (2012). Short communications: Seroprevalence of *Neospora caninum*, paratuberculosis and Q fever in cattle in Belgium in 2009 – 2010. Vet. Rec. 171: 477.

van Hulzen, K.J.E., Nielen, M, Koets, A.P., de Jong, G., van Arendonk, J.A.M. & Heuven, H.C.M. (2011). Effect of herd prevalence on heritability estimates of antibody response to *Mycobacterium avium* subspecies *paratuberculosis.* J. Dairy Sci. 94: 992-997.

van Hulzen, K.J.E., Schopen, G.C.B., van Arendonk, J.A.M., Nielen, M., Koets, A.P., Schrooten, C. & Heuven, H.C.M. (2012). Genome-wide association study to identify chromosomal regions associated with antibody response to *Mycobacterium avium* subspecies *paratuberculosis* in milk of Dutch Holstein-Friesians. J. Dairy Sci. 95: 2740-2748.

van Hulzen, K.J.E., Koets, A.P., Nielen, M., Heuven, H.C.M., van Arendonk, J.A.M. & Klinkenberg, D. (2014). The effect of genetic selection for Johne's disease resistance in dairy cattle: Results of a genetic-epidemiological model. J. Dairy Sci. 97: 1762-1773.

van Roermund, H.J.W., Bakker, D., Willemsen, P.T.J. & de Jong, M.C.M. (2007). Horizontal transmission of *Mycobacterium* avi*u*m subsp. *paratuberculosis* in cattle in an experimental setting. Calves can transmit the infection to other calves. Vet. Microbiol. 122: 270-279.

Verschoor, C.P., Pant, S.D., You, Q., Schenkel, F.S., Kelton, D.F. & Karrow, N.A. (2010). Polymorphisms in the gene encoding bovine interleukin-10 receptor alpha are associated with *Mycobacterium avium* ssp. *paratuberculosis* infection status. BMC Genet. 11: 23.

Vidal, S.M., Malo, D., Vogan, K., Skamene, E. & Gros, P. (1993). Natural Resistance to Infection with Intracellular Parasites: Isolation of a Candidate for *Bcg*. Cell 73: 469-485.

VIT (2013). Beschreibung der Zuchtwertschätzung für Milchleistungsparameter, Zellzahl, Exterieurmerkmale, Nutzungsdauer und Zuchtleistungsmerkmale.Stand: Dezember 2013. http://www.vit.de/fileadmin/user_upload/vit-fuers-rind/zuchtwertschaetzung/milchrinder-zws-online/Zws_Bes_deu.pdf. Dowloaded 20.12.2013.

Weber, M.F. & Schaik, G. (2007). Results of Dutch bulk milk quality assurance programme for paratuberculosis. Proceedings of the 9th International Colloquium on Paratuberculosis, 29.10.-02.11.2007,Tsukuba, Japan, p. 324-327.

Weber, M.F., Lam, T.J.G.M. & Franken, P. (2009). Milk quality assurance for paratuberculosis in the Netherlands. Proceedings of the 10th International Colloquium on Paratuberculosis, 09.-14.08.2009, Minneapolis, Minnesota USA, p. 233-234.

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.

Whittington, R.J., Marsh, I., Turner, M.J., MacAllister, S., Choy, e., Eamens, G.J., Marshall, D.J. & Ottaway, S. (1998). Rapid detection of *Mycobacterium paratuberculosis* in clinical samples from ruminants and in spiked environmental samples by modified BACTEC 12B radiometric culture and direct confirmation by IS*900* PCR. J. Clin. Microbiol. 36: 701-707.

Whittington, R.J. & Windsor, P.A. (2009). In utero infection of cattle with *Mycobacterium avium* subsp*. paratuberculosis:*A critical review and meta-analysis. Vet. J. 179: 60-69.

Windsor, P.A. & Whittington, R.J. (2010). Evidence for age susceptibility of cattle to Johne's disease. Vet. J. 184: 37-44.

Wu, S.W., Pao, C.C., Chan, J. & Yen, T.S. (1991). Lack of mycobacterial DNA in Crohn's disease tissue. Lancet 337: 174-175.

Wu, C., Livesey, M., Schmoller, S.K., Manning, E.J.B., 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.

Yildirim, D. & Civelek, T. (2013). Prevalence of Subclinical Paratuberculosis in Dairy Cattle in Uşak Region. Kafkas Univ. Vet. Fak. Derg. 19: 121-126.

Zhu, M. & Zhao, S. (2007). Review: Candidate gene identification approach: progress and challenges. Int. J. Biol. Sci. 3: 420-427.

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Ich erkläre:

Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe.

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