

**Distribution of *Mycobacterium avium* subspecies  
*paratuberculosis* in clinically asymptomatic bulls and different  
non-ruminant species**

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**SUMMARY**

Paratuberculosis is a disease of global distribution, which mainly occurs in ruminants. The disease is triggered by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which causes a chronic, degenerative granulomatous inflammation of the intestinal tract, in turn leading to diarrhoea, weight loss, reduced reproductive performance, and finally death. The economic losses sustained to the agricultural sector, as well as the potential aetiological link between MAP and Crohn's disease in humans, are the main reasons for the indispensability of an efficient control of paratuberculosis. The main challenge in ensuring efficient disease control is that infected animals do not necessarily show clinical symptoms and diagnostic methods are not reliable at this stage of the disease. To examine the unrecognized spread of asymptotically infected hosts as well as potential transmission pathways, three studies were conducted in this thesis. In an additional study, a recombinase polymerase amplification (RPA) assay was developed to enable the detection of MAP in low-resource settings or directly in the stable.

The occurrence of MAP in the faeces, blood, and semen of a naturally infected, but clinically asymptomatic German Fleckvieh bull (related to Simmental: *Bos primigenius taurus*) was analysed over a period of four years, from the age of seven weeks until necropsy. MAP was intermittently detected by IS-900 based semi-nested PCR, real-time PCR, and bacteriological cultivation, with occurrence of MAP-free intervals of up to nine weeks. A recurring detection pattern was not found. Besides antigen detection, most of the implemented control programmes are based on the detection of an antibody response by ELISA. However, the examined bull was a serologically negative shedder over the entire investigation period. Such animals are one of the main reasons for the reduced success of paratuberculosis control programmes.

As well as ruminants, non-ruminants can be infected with MAP, but currently the knowledge concerning potential hosts is incomplete. It has been known for many years that the faeces of infected non-ruminants contains concentrations of up to  $10^6$  MAP cells/g and might therefore play a role in the transmission of MAP to ruminants, through shared habitats. In this thesis, the presence of MAP was confirmed in rock hyraxes (*Procapra capensis*); a common marmoset (*Callithrix jacchus*); and a cottontop tamarin (*Saguinus oedipus*). Three of the investigated rock hyraxes were captured in South Africa and their four descendants were subsequently born in a quarantine stable at a German zoological garden. PCR-analysis of a pooled faecal sample of all animals, and

subsequent examination of individual faeces, confirmed the excretion of MAP by two rock hyraxes. In contrast, MAP was not detected in the faeces of 20 non-human primates (NHP), belonging to seven different species, although the presence of MAP was confirmed in the ileum of one cottontop tamarin (*Saguinus oedipus*), and the bone marrow of a common marmoset (*Callithrix jacchus*). Analogous to the examination of the NHP, the distribution of MAP within the body of the host was also investigated in the bull and the rock hyraxes. MAP manifestation within most body regions of clinically asymptomatic ruminants as well as non-ruminants was observed, including in the tissues of the gastrointestinal tract, urogenital tract, cardiovascular system, and/or respiratory system. Since none of the investigated animals (the bull; the group of rock hyraxes; or either of the non-human primate species) showed typical paratuberculosis clinical symptoms or lesions after necropsy, an asymptomatic infection with MAP was assumed. Therefore, it can be expected that MAP replicates within the asymptotically infected host without triggering typical pathohistological changes or clinical signs of paratuberculosis. Furthermore, it is reasonable to suspect that non-ruminants excrete the pathogen intermittently and in different concentrations, as clinically asymptomatic ruminants do.

In order to control a disease, it is necessary to discover all routes of transmission. Although since 1970 it has been known that MAP is present in the semen and the reproductive organs of bulls with clinical symptoms and/or paratuberculosis-typical pathological changes, only a small number of studies have scrutinised the role of bulls in the transmission of MAP. In this thesis, the presence of MAP was confirmed in the semen and reproductive organs of an asymptomatic infected bull. These results support the hypotheses that MAP might be transmitted to cows via semen under natural mating conditions, or, likely, by artificial insemination; however, the influence of the semen extender on the viability of MAP is unknown. In addition to the investigated bull, MAP was also observed in the reproductive organs of one female and one male rock hyrax. Interestingly, female animals of this species have a haemoendothelial placenta, meaning the foetuses have direct contact with the maternal blood, and that vertical transmission of MAP might also, therefore, be a possibility in non-ruminants.

Another interesting finding of this thesis, was the detection of MAP in the bone marrow of the investigated bull and the common marmoset. As it has been demonstrated that a MAP-related pathogen, *Mycobacterium tuberculosis*, can persist within the

mesenchyme cells of the bone marrow in a viable, but not culturable, stage, it was concluded that the bone marrow might be a niche for the establishment of latent tuberculosis infection. Therefore, it might also be assumed that bone marrow plays a key role in the establishment of latent paratuberculosis infection.

Currently, available detection methods (PCR, cultivation, ELISA) require sophisticated laboratory equipment; to allow the identification of MAP-infected animals in low-resource settings or directly in the stable, a simple molecular diagnostic tool is needed. In order to fill this gap, an RPA assay targeting the MAP IS900 gene was developed. In contrast to commonly used diagnostic methods (culture and real-time PCR), RPA assays are extremely fast (3-15 minutes), easy to perform, run at a constant temperature of 42°C, and all reagents are cold-chain independent. The developed MAP-RPA assay has a limit of detection of 16 DNA molecules, and does not show any cross-reactivity to other bacterial strains of clinical importance. The clinical specificity and sensitivity is calculated at 100% (n=20) and 90% (n=48), respectively, based on comparison with a well-established real-time PCR.

In conclusion, the studies performed for this thesis highlight the potential risk of MAP transmission from asymptomatic animals. Ruminants as well as non-ruminants play an important role in the silent spread of MAP within an animal stock. The obtained results confirm the high occurrence rate of MAP in semen and the male reproductive organs. Thus, bulls might be a part of the transmission cycle of MAP to cows and their descendants or fetuses. Furthermore, non-ruminants seem to be more susceptible to MAP than commonly believed. For the first time, the presence of MAP in rock hyraxes and the common marmoset was confirmed. Further studies should address the pathogenesis of clinically asymptomatic infected ruminants and non-ruminants, and routes of transmission via semen as well as between different species.



## **1 GENERAL INTRODUCTION**

***MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (MAP)****TAXONOMY**

The genus *Mycobacterium* and the closely related *Corynebacterium*, *Nocardia* and *Rhodococcus* are members of the order *Actinomycetales*. *Mycobacterium* belongs to the *Mycobacteriaceae* family (Table 1). Although MAP was once considered a separate species, current taxonomy ranks MAP to the *Mycobacterium avium-complex* (MAC) (Turenne and Alexander, 2010).

**Table 1:** Taxonomy of MAP (NCBI Taxonomy Browser, 2017)

<b>ORGANISM TAXONOMY</b>	
<b>Kingdom</b>	Bacteria
<b>Phylum</b>	Actinobacteria
<b>Class</b>	Actinobacteria
<b>Order</b>	Corynebacteriales
<b>Family</b>	Mycobacteriaceae
<b>Genus</b>	Mycobacterium
<b>Species</b>	Mycobacterium avium

The genus *Mycobacterium* consists of three major groups: *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, and all other non-tuberculosis mycobacteria, also called ‘Mycobacteria other than tuberculosis’ (MOTT). Currently, over 150 MOTTs have been officially described (Tortoli, 2014). Traditionally, the classification schema for MOTT has been based on phenotypic characteristics. MOTTs were divided into four Runyon Groups (I-IV) on the basis of features such as growth rate, growth temperature, pigmentation and biochemical activities (Rolle and Mayr, 2002). *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium asiaticum* and *Mycobacterium simiae* are some agents found in Runyon Group I. Characteristics of Runyon Group I mycobacteria are their slow growth rate and distinctive pigmentation (bright yellow in white light). Mycobacteria belonging to Runyon Group II also have a slow growth rate, but are pigmented yellow in white light and orange in the dark. Agents of this group

include *Mycobacterium scrofulaceum*, *Mycobacterium gordonae*, *Mycobacterium szulgae*, *Mycobacterium xenopi*, and *Mycobacterium flavescens*. Slowly growing, photochromogenic mycobacteria belong to Runyon Group III. This group includes *Mycobacterium ulcerans*, *Mycobacterium haemophilum* and *Mycobacterium malmoeense*. Although some strains of the *Mycobacterium avium* complex (MAC) have bright yellow pigmentation, they also belong to Runyon Group III. MAC species include *Mycobacterium intracellulare*, *Mycobacterium avium* subsp. *avium* (MAA), *Mycobacterium avium* subsp. *hominissuis* (MAH), *Mycobacterium avium* subsp. *silvaticum* (MAS) and *Mycobacterium avium* subsp. *paratuberculosis* (MAP). All fast-growing mycobacteria (within seven days) belong to Runyon Group IV; this group includes species such as *Mycobacterium fortuitum*, *Mycobacterium abscessus* and *Mycobacterium chelonae*.

To date, MAC species have been characterised on the basis of phenotypic features and genetic analyses. Phylogenetic trees of mycobacteria were mostly designed on the basis of complete 16S rRNA and/or of amino acid sequences by sequencing DNA fragments of the *hsp65* and *rpoB* genes. Currently, ten species of slow-growing mycobacteria and a subset of “MAC-other” isolates with an undetermined classification belong to the MAC. These closely related mycobacteria are distinguished by host preference, degrees of pathogenicity and environmental distribution (Table 2).

**Table 2:** The *Mycobacterium avium complex* (MAC). Modified from Rindi and Garzelli (2014)

Species	Sub-species	Pathogenicity	Host preference	Reference
<i>M. avium</i>	Subsp. <i>avium</i>	Avian TB	Birds	Mijs et al. (2002)
	Subsp. <i>hominissuis</i>	Pulmonary infections, cervical lymphadenitis, soft tissue infections disseminated infections	Human, porcine	Mijs et al. (2002)
	Subsp. <i>paratuberculosis</i>	Johne's disease	Ruminants	Thorel et al. (1990)
	Subsp. <i>silvaticum</i>	TB-like disease	Wood pigeons	Thorel et al. (1990)
<i>M. intracellulare</i>		Pulmonary infections	Human	Pfyffer et al. (2015)
<i>M. indicus pranii</i>		Pulmonary infections	Human	Saini et al. (2009)
<i>M. colombiense</i>		Disseminated infections, lymphadenopathy	Human	Murcia et al. (2006)
<i>M. chimaera</i>		Pulmonary infections	Human	Tortoli et al. (2004)
<i>M. marseillense</i>		Pulmonary infections	Human	Ben Salah et al. (2009)
<i>M. timonense</i>		Pulmonary infections	Human	Ben Salah et al. (2009)
<i>M. bougherdurhonense</i>		Pulmonary infections	Human	Ben Salah et al. (2009)
<i>M. vulneris</i>		Lymphadenopathy, wound	Human	van Ingen et al. (2009)
<i>M. arosiense</i>		Osteomyelitis	Human	Bang et al. (2008)
<i>M. yongonense</i>		Pulmonary infections	Human	Kim et al. (2013)
"MAC-others"		Pulmonary infections	Human	Smole et al. (2002)

Currently, four subspecies belong to *M. avium*, including *M. avium* subsp. *avium* (MAH), *M. avium* subsp. *paratuberculosis* (MAP), *M. avium* subsp. *silvaticum* (MAS) and *M. avium* subsp. *hominissuis* (MAH). Although these subspecies were taxonomically closely related mycobacteria, each has a specific pathogenicity and host range (Rindi and Garzelli, 2014). Recently, availability of complete genome sequences and the presence of different insertion sequences (IS) among the *M. avium* subspecies have provided the opportunity to differentiate these subspecies by molecular biological typing methods. The distinction between the *M. avium* subspecies is largely based on IS1245, IS1311, IS900 and IS901 (Rindi and Garzelli, 2014).

#### PATHOGEN CHARACTERIZATION AND TENACITY

MAP is an acid-fast, non-moving, Gram-positive rod that is 0.5-2.0 µm long and 0.3-0.5 µm wide (Manning and Collins, 2001). This pathogen grows under aerobic conditions and has a generation interval of 1.3-4.4 days (Lambrecht et al., 1988). A further characteristic of MAP is its mycobactin-dependent growth, which must be considered during bacteriological cultivation and is used as an exclusion criterion in the differentiation from other mycobacteria.

Free, long-chain fatty acids in the cell wall make the bacterium resistant to acid and alcohol. The cell wall structure is divided into three different layers (Daffe and Draper, 1998). The most outer part is a mycolic acid-containing layer with a membrane-like structure underneath lies the murein layer followed by the inner-most plasma membrane. Through the use of intercellular filaments, MAP is able to form bacterial nests, known as clusters (Merkal, 1973).

Due to this special cell wall structure, MAP is able to survive for long durations in the environment. MAP can survive for up to 270 days in the faeces of cattle (Larsen et al., 1956). On contaminated pastures, the pathogen is able to survive for up to 11 months (Gay and Sherman, 1992). Very sensitive MAP responds only to UV radiation (Whittington et al., 2004). The pathogen can thus survive on grass-covered pastures. Here, it is protected against UV radiation for up to 32 weeks; on completely bare, grazed sheep pastures for up to two weeks. Another influence on the survival of MAP is soil pH (Ward and Perez, 2004; Whittington et al., 2004).

MAP also has a very high thermal stability. MAP can survive at both very low and very high temperatures. Cultivation of MAP from the naturally contaminated faeces of cattle remains possible after storage for 12 months at -14 °C (Larsen et al., 1956). Its particularly high heat stability is especially important for food production. It has been demonstrated that viable MAP cells remain detectable in pasteurized milk (Grant et al., 1998). The pathogen is also not completely killed during the production of cheese (Spahr and Schaefroth, 2001).

Due to this high durability, the range of effective disinfectants is limited. Faeces and dirt often surround the pathogen; therefore, most common disinfectants are ineffective. To date, only formaldehyde-containing disinfectants (Chiodini et al., 1984a) and peracetic acid agents (chemical sterilization) have been proven effective.

## GENOTYPES

On the base of different molecular techniques, diverse strain types of MAP have previously been detected. Collins et al. (1990) proved that two major groups of MAP strains exist, which were named after the first known host species. The “Type S” strains (sheep-type) were detected in sheep. This strain is characterized by an extremely slow growth (up to 12 months), and isolates are usually pigmented. In contrast, isolates belonging to the “Type C” strains (cattle-type) show growth within 5-16 weeks, and are unpigmented. Isolates of this type were often observed in cattle, but were also detected in a wide range of other non-ruminant animals (Ghadiali et al., 2004; Hutchings et al., 2010; Motiwala et al., 2004). Until recently, it was thought that a third group of MAP strains existed, the termed “Type III” or “intermediate” strains. It was expected that “Type III” strains were intermediate strains between the Type S and Type C strains; however, whole sequence data of these isolates has demonstrated that no Type S strains exist, but that such isolates are actually a subtype of the Type S strains (Stevenson, 2015). Another group of MAP strains are the “Bison” or “B-type” strains. This strain type was differentiated by IS1311 PCR-REA (restriction enzyme analysis) on the basis of the number of IS1311 copies with C/T polymorphisms at position 223. Furthermore, B-type isolates from India are distinguishable from American isolates by a TG deletion at positions 64 and 65 of the IS1331 at locus 2 (Sohal et al., 2013). For this reason, these strains are grouped into the “Indian Bison Type,” which is a sub-lineage of Type C strains (Thibault et al., 2012).

## GENOME

In 2005, the first whole genome sequence (WGS) of a MAP isolate (MAP strain K10; GenBank No. AE016958) was published. The analysis performed of the Type II strain yielded a single circular sequence of 4,829,781 base pairs, with a G+C content of 69.3% (Li et al., 2005). After optical mapping and resequencing of the MAP strain K10 genome by the Illumina sequencing platform, an edited sequence and annotation was issued. To date, the WGS of five different MAP isolates have been published. These isolates originated from the USA, Egypt and India, and were isolated from cattle as well as from one human breast milk sample. WGS data demonstrates that the genome size of MAP ranges from 4.78 Mb to 4.83 Mb. The G+C content of all five isolates is 69.30%. The number of genes ranges from 4,446 to 4,464, and the number of proteins ranges from 4,153 to 4,321. Furthermore, sequence data of a further 34 MAP isolates are presently housed in the NCBI databank (Table 3). These isolates come from varying hosts, such as cattle, sheep, goat, camel, human and red deer. Moreover, these isolates were isolated in different countries, including for example the USA, India and China.

**Table 3:** Genome assemblies and annotation reports of *Mycobacterium avium* subsp. *paratuberculosis* (NCBI; 12.04.2017)

Strain	Host	Country of origin	Level	Size (Mb)	GC%	Number of scaffolds	Genes	Proteins	Accession number
K-10	Cattle	USA	Complete	4.83	69.30	-	4,457	4,289	NC_002944.2
MAP4	Human	n.s.	Complete	4.83	69.30	-	4,463	4,321	NC_021200.1
E1	Cattle	Egypt	Complete	4.78	69.30	-	4,446	4,153	NZ_CP010113.1
E93	Cattle	Egypt	Complete	4.79	69.30	-	4,453	4,228	NZ_CP010114.1
MAP/TANUVAS/ TN/India/2008	Cattle	India	Complete	4.83	69.30	-	4,464	4,296	NZ_CP015495.1
CLIJ644	Cattle	Australia	Contig	4.71	69.20	636	636	4,581	NZ_AFN000000000.1
CLIJ361	Sheep	Australia	Contig	4.61	69.00	1,147	4,672	3,800	NZ_AFNS000000000.1
CLIJ623	Cattle	Australia	Contig	4.52	69.00	915	4,576	3,839	NZ_AFH000000000.1
Pt139	Human	Australia	Contig	4.65	69.10	752	4,584	3,987	NZ_AFPC000000000.1
Pt144	Human	Australia	Contig	4.58	69.00	838	4,540	3,945	NZ_AFPD000000000.1
Pt145	Human	Australia	Contig	4.57	69.00	916	4,520	3,899	NZ_AFPE000000000.1
Pt146	Human	Australia	Contig	4.58	69.00	955	4,584	3,884	NZ_AFPF000000000.1
Pt154	Human	Australia	Contig	4.50	68.90	1,077	4,526	3,774	NZ_AFPG000000000.1
Pt155	Human	Australia	Contig	4.56	69.00	957	4,540	3,880	NZ_AFPH000000000.1

n.s.: Not specified



Table 3: continued

Strain	Host	Country of origin	Level	Size (Mb)	GC%	Number of scaffolds	Genes	Proteins	Accession number
Pt164	Human	Australia	Contig	4.67	69.20	592	4,559	4,059	NZ_AFP100000000.1
S397	Sheep	USA	Contig	4.81	69.30	176	4,516	4,245	NZ_AFIF000000000.1
JQ5	Camel	Saudi Arabia	Contig	4.74	69.10	176	4,495	3,544	NZ_AHAZ000000000.1
JQ6	Camel	Saudi Arabia	Contig	4.70	69.00	176	4,485	3,161	NZ_AHBA000000000.1
JTC 1281	Oryx	n.s.	Contig	4.76	69.30	1,030	4,695	4,008	NZ_AGAK000000000.1
JTC 1285	Goat	n.s.	Contig	4.75	69.30	1,115	4,710	4,002	NZ_AGAL000000000.1
4B	Human	n.s.	Contig	4.74	69.30	479	4,568	4,123	NZ_AGAM000000000.1
DT 3	British red deer	n.s.	Contig	4.73	69.30	531	4,575	4,115	NZ_AGAN000000000.1
Env 210	Cattle farm	n.s.	Contig	4.74	69.30	538	4,558	4,127	NZ_AGAO000000000.1
DT 78	Water buffalo	n.s.	Contig	5.00	69.20	1,201	4,910	4,142	NZ_AGAP000000000.1
ATCC 19698	Cattle	n.s.	Contig	4.74	69.30	1,092	4,732	3,992	NZ_AGAR000000000.1
S5	Goat	India	Contig	4.80	69.20	178	4,481	3,844	NZ_ANPD000000000.1
08-8281	Sheep	USA	Contig	4.88	69.20	465	4,594	3,030	NZ_AYLY000000000.1
10-4404	Cattle	USA	Contig	4.81	69.30	546	4,640	3,558	NZ_AYNR000000000.1

n.s.: Not specified

**Table 3:** continued

<b>Strain</b>	<b>Host</b>	<b>Country of origin</b>	<b>Level</b>	<b>Size (Mb)</b>	<b>GC%</b>	<b>Number of scaffolds</b>	<b>Genes</b>	<b>Proteins</b>	<b>Accession number</b>
10-5864	Cattle	USA	Contig	4.81	69.30	567	4,617	3,314	NZ_AYNV000000000.1
10-5975	Bison	USA	Contig	4.63	69.00	1,256	4,566	3,384	NZ_AYNW000000000.1
11-1786	Sheep	USA	Contig	4.67	69.00	1,308	4,647	3,475	NZ_AYOA000000000.1
2015WD-1	Cattle	China	Scaffold	4.75	69.30	259	4,607	4,209	NZ_LKUS000000000.1
2015WD-2	Cattle	China	Scaffold	4.73	69.30	240	4,607	4,198	NZ_LKUT000000000.1
NL 89C	Cattle	Canada	Contig	4.78	69.30	98	4,449	4,279	NZ_LGRY000000000.1
NL 93B	Cattle	Canada	Contig	4.77	69.30	90	4,441	4,278	NZ_LGRZ000000000.1
NL 96E	Cattle	Canada	Contig	4.77	69.30	90	4,444	4,280	NZ_LGSD000000000.1
NL 95A	Cattle	Canada	Contig	4.78	69.30	94	4,445	4,280	NZ_LGSA000000000.1
NL 95B	Cattle	Canada	Contig	4.77	69.30	90	4,444	4,281	NZ_LGSB000000000.1
NL 95E	Cattle	Canada	Contig	4.77	69.30	97	4,447	4,271	NZ_LGSC000000000.1

n.s: Not specified

## PARATUBERCULOSIS

*Mycobacterium avium* ssp. *paratuberculosis* (MAP) is the infectious agent of Johne's disease. The disease is characterized by a degenerative chronic granulomatous inflammation of the intestinal tract, causing diarrhoea, weight loss, reduced reproductive performance, and eventually death (Harris and Barletta, 2001). In general, an infection with MAP occurs in domestic ruminants; however, the pathogen has also been detected in a wide range of other domesticated, wild, and zoo animals, including non-ruminants (Beard et al., 2001a; Greig et al., 1999; Münster et al., 2013a). In accordance with the Ordinance on Notifiable Animal Diseases (Anonymous, 2011), paratuberculosis is a notifiable disease in Germany. Clinical cases of the disease are recorded via the *Tierseuchen Nachrichten System* (Animal Disease News System).

## HISTORY OF PARATUBERCULOSIS

Initial reports of the occurrence of paratuberculosis were reported in the mid-1820s, but the cases were not well described and the cause was unknown. The initial clear description of a paratuberculosis case was reported in 1895 by Dr. Heinrich Albert Johne and Dr. Landedon Fortingham (Johne and Fortingham, 1895). The authors observed thickened intestinal mucosa and enlarged mesenteric lymph nodes during the necropsy of a cow from Oldenburg (Germany). In the inflamed tissue, numerous of acid-fast (red-staining) bacteria were found. As the tuberculosis-like bacterium did not elicit disease in guinea pigs and failed to grow in standard conditions used for *Mycobacterium tuberculosis*, it was concluded that the detected pathogen was the same as that which caused tuberculosis in birds. The clinical picture was coined pseudotuberculosis enteritis due to similarities in pathological changes, such as intestinal tuberculosis.

After the first report of pseudotuberculosis enteritis, several reports of the new disease began to be recounted from Europa and the USA. In 1906, Professor Bernhardt Bang discovered that cattle that became sick with the new disease did not show an immune response to *Mycobacterium bovis* tuberculin antigens, whereas *Mycobacterium avium* tuberculin did trigger an immune reaction (Bang, 1906). The first cultivation of the pathogen causing this new disease occurred by way of a laboratory accident. Contamination with *Mycobacterium phlei* provided essential nutrients for the growth of

the then-named *Mycobacterium enteritidis chronicae pseudotuberculosis bovis* (Twort and Ingram, 1912). Now that cultivation of the pathogen was possible, diagnostic tests were able to be established. As had been done for tuberculosis, a so-called ‘johnin tuberculin,’ to be used in skin testing, was developed. Furthermore, complement fixation and agglutination techniques evolved to detect an antibody reaction in serum samples.

In 1920, cases of paratuberculosis also began to be reported in Africa and Asian. From this moment, it became obvious that paratuberculosis had spread worldwide. The first official name of the aetiological agent was *Mycobacterium paratuberculosis*, which was published in *Bergey’s Manual of Determinative Bacteriology* in 1923. Since 1940, it has been known that paratuberculosis is not only a disease of domesticated livestock; rather, wildlife can also be affected.

It must be noted that data published between 1920 and the 1970s was performed with the working strain ATCC 12227, also called strain 18. Today, it is known that strain 18 was a laboratory contamination and not a *Mycobacterium paratuberculosis*, but rather a strain of *Mycobacterium avium* serovar 2 (Chiodini, 2005).

The first time the pathogen was isolated from a human was in 1984 from a patient with Crohn’s disease. Although it was long assumed that a link between *Mycobacterium paratuberculosis* and Crohn’s disease exists, this was the first physical evidence of the link. Essential for the molecularbiological detection of the pathogen was the detection of the insertion element (IS) 900 by two independent research groups (Green et al., 1989; McFadden et al., 1987). After several name changes, the pathogen was renamed, based on DNA-DNA hybridization analyses (Thorel et al., 1990), to *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

## PATHOGENESIS

The pathogenesis of paratuberculosis remains incompletely understood. Immune status, age, the pathogen concentration ingested and the resistance profile of the affected animal all play important roles (Dirksen et al., 2003) in the course of the infection. Whitlock and Buergelt (1996) divided the disease course of paratuberculosis into four stages:

STAGE I: “Silent” infection of calves, young livestock and adults

STAGE II: Subclinical disease of carrier adults

STAGE III: Clinical disease

STAGE IV: Advanced clinical disease.

STAGE I: “Silent” infection of calves, young livestock and adults

Calves and young animals are generally infected via the faecal-oral route, through the ingestion of faeces or milk/colostrum contaminated with MAP. After oral ingestion, the pathogens enter the digestive tract and are transported by M-cells into the intestinal epithelium (Buergelt, 1991; Momotani et al., 1988). After the manifestation of MAP in the intestinal epithelium, the pathogen is transported to the lymphoid tissue (Peyer’s patches) of the intestine, to the mesentery and via the thoracic duct into other organs (Chiodini and Rossiter, 1996). The subepithelial and intraepithelial macrophages are the target cells for MAP. A cell-mediated immune response of the host is encouraged (Chiodini et al., 1984a; Clarke, 1997). However, the pathogen is able to survive these cellular defence mechanisms.

STAGE II: Subclinical disease of carrier adults

At this stage, MAP commences intracellular replication within the macrophages. The pathogen is then either excreted in the faeces or phagocytosed by other macrophages. With pathogen release, the cellular immune response changes into the humoral immune response (Chiodini et al., 1984a). Pathomorphology shows low-to-moderate mucosal hypertrophy and the noticeable enlargement of mesenteric lymph nodes. At this stage, the infected animal shows no clinical symptoms.

### STAGE III: Clinical disease

Infected animals first develop clinical symptoms at an age of between two and five years (Larsen et al., 1975; Valentin-Weigand and Goethe, 1999). During this stage, the feed intake and general condition of the animal initially remains undisturbed (Rosenberger, 1978). Weight loss, diarrhoea, and a decrease in milk production and fattening performance are the first signs of the disease observed. Humoral immunity predominates during this stage of the disease (Chiodini et al., 1984a), and the detection of antibodies is possible.

### STAGE IV: Advanced clinical disease

In the final stage, diseased animals show massive clinical signs. These animals suffer extreme weight loss, despite undisturbed feed intake (Figure 1). Another feature is recurring watery, foul-smelling diarrhoea. With disease progression, general health is increasingly disturbed (Dirksen et al., 2003). Oedema, cachexia and serous atrophy of body fat occur in severe cases (Clarke, 1997; Olsen et al., 2002). Pathologically, it is noticeable that the mucosa of the ileum, jejunum and colon has a high degree of hypertrophy (Figure 2). The mesenteric lymph nodes are also severely enlarged. In the final stage, the humoral immune response decreases to anergy (Chiodini, 1996).



**Figure 1:** Dairy cattle in the final stage of paratuberculosis (Division of Microbiology and Animal Hygiene, Georg-August-University Göttingen)



**Figure 2:** Pathological findings of paratuberculosis (Division of Microbiology and Animal Hygiene, Georg-August- University Göttingen)

Primary MAP is present in the tissues of the gastrointestinal tract and appending lymph nodes (Sweeney et al., 1992b). In a study with tissue samples from 131 cattle collected in a slaughterhouse, MAP was detected most frequently (77.6%) in the tissues of the gastrointestinal tract (Hasonova et al., 2009). In addition, many studies have reported the occurrence of MAP in tissues other than gastrointestinal tract (Table 4) or reproductive system of cows and heifers (Table 5) and bulls (Table 6). It is likely that MAP is disseminated within the host in macrophages via the blood or lymphatic system (Clarke, 1997). It was originally thought that the spread of MAP within the host occurs during the advance disease stage (Chiodini, 1996); however, other studies have reported that the pathogen is spared as early as the subclinical infection stage (Buergelt et al., 1978; Condrón et al., 1994). A recent study concluded that the dissemination of MAP within the host take place soon after infection (Mortier et al., 2013).

**Table 4:** Distribution of MAP in extraintestinal tissues other than the reproductive tract of infected cattle

Specimens	References
Liver and hepatic lnn.	Antognoli et al. (2008); Collins (1997); Hasonova et al. (2009); Khol et al. (2010); Pavlik et al. (2000)
Retropharyngeal lnn.	Pavlik et al. (2000)
Mandibular lnn.	Pavlik et al. (2000)
Spleen	Collins (1997); Hasonova et al. (2009); Pavlik et al. (2000)
Lung and lnn.	Antognoli et al. (2008); Collins (1997); Hasonova et al. (2009); Hasonova et al. (2009); Pavlik et al. (2000)
Kidneys	Antognoli et al. (2008); Collins (1997)
Popliteal lnn.	Antognoli et al. (2008)
Prescapular lnn.	Antognoli et al. (2008)
Heart	Antognoli et al. (2008)
Blood	Collins (1997); Buergelt and Williams (2004); Buergelt et al. (2004); Juste et al. (2005); Khol et al. (2010); Münster et al. (2013b)

lnn: Lymph nodes



**Table 5:** Distribution of MAP in the reproductive system of cows and heifers

<b>Specimens</b>	<b>References</b>
Udder	Collins (1997); Doyle (1954); Hasonova et al. (2009); Merkal (1984)
Supramammary Inn.	Alexejeff-Goleff (1929); Antognoli et al. (2008); Hasonova et al. (2009); Pavlik et al. (2000); (Sweeney et al., 1992a)
Milk	Doyle (1954); Buergelt and Williams (2004); Smith (1960); Sweeney et al. (1992b); Taylor et al. (1981)
Uterus	Collins (1997); Hasonova et al. (2009)
Foetus	Pearson and McClelland (1955); Merkal (1984); Seitz et al. (1989); Sweeney et al. (1992b)
Foetal membranes	Doyle (1958)
Cotyledons	Doyle (1958); Lawrence (1956); Pearson and McClelland (1955);
Uterine flush	Pribylova et al. (2013); Rohde and Shulaw (1990)
Vaginal flush	Pribylova et al. (2013)
Follicular fluid	Pribylova et al. (2013)
Inn: Lymph nodes	

**Table 6:** Distribution of MAP in the reproductive system of bulls

Specimens	References
Testes	Ayele et al. (2004); Glawischnig et al. (2004); Tunkle and Aleraj (1965)
Bulbourethral gland	Larsen and Kopecky (1970); Tunkle and Aleraj (1965)
Epididymis	Ayele et al. (2004); Collins (1997); Khol et al. (2010)
Seminal vesicle	Ayele et al. (2004); Khol et al. (2010); Larsen and Kopecky (1970); Larsen et al. (1981)
Prostate gland	Larsen and Kopecky (1970); Larsen et al. (1981)
Semen	Ayele et al. (2004); Buergelt et al. (2004); Eppleston and Whittington (2001); Khamesipour et al. (2014); Khol et al. (2010); Larsen and Kopecky (1970); Larsen et al. (1981); Münster et al. (2013b); Sharifzadeh et al. (2010); Tunkle and Aleraj (1965)

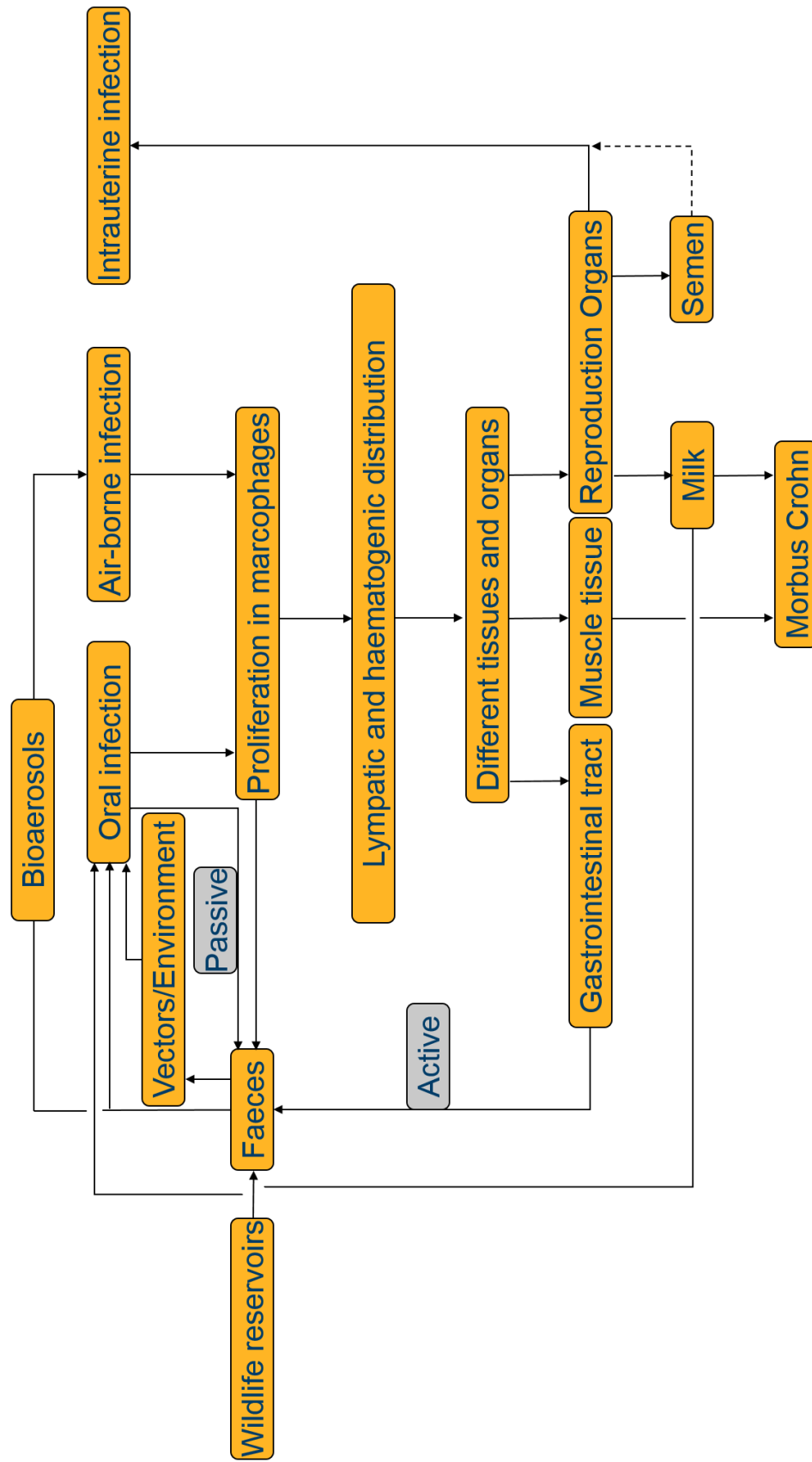
## SUSCEPTIBILITY AND TRANSMISSION

Calves younger than 30 days appear to be the most susceptible to a MAP infection (Clarke, 1997; Larsen and Kopecky, 1970). Neonates are considered to be at especially high risk of becoming infected with MAP. The cause of the increased susceptibility of newborn animals is currently unknown; however, as Peyer's patches have been observed to be the major point of entry for MAP (Momotani et al., 1988; Sigurdardottir et al., 2005), it is assumed that the peak of activity of Peyer's patches seen in neonates may play an important role in their susceptibility. Cattle over two years of age are considered to be resistant to infection; however, an infection with MAP can occur at all ages.

An infection with MAP does not only depend on age. Other factors, such as infectious dose of MAP, pathogen virulence, stress factors or the genetic profile of the host also have an impact on infection susceptibility and the disease course. It is assumed that an infection with MAP occurs in approximately 85% of the subsequently infected animals within the first weeks of life, 10% of the animals are infected *in utero* and only 5% of the animals are infected within the first year of life. To trigger an infection, a minimum

infectious dose of  $10^3$  pathogens per calf is required (Gerlach, 2002). In experimental studies, a relationship between the infectious dose and the development of lesions and clinical signs has been reported (Begg and Whittington, 2008). Furthermore, it was observed that earlier disease development occurs after larger doses of MAP. Furthermore, stress factors such as intensive livestock production, poor feeding, transport and calving may also influence the disease course (Chiodini et al., 1984a). Differences between breeds in the incidence of infection with MAP and disease exist. The clinical picture of paratuberculosis appears to occur more frequently in Jersey or Guernsey cattle, in comparison to other breeds (Cetinkaya et al., 1997; Jakobsen et al., 2000). In the literature, the heritability ( $h^2$ ) of an infection with MAP in dairy cattle has been estimated to range from 0.09-0.12 (Gonda et al., 2006; Hinger et al., 2008; Koets et al., 2000; Mortensen et al., 2004). Several candidate genes that were considered to have an impact on host susceptibility to MAP in cattle have also been studied.

MAP can be transmitted vertically and horizontally to susceptible hosts. The theoretical pathways of MAP transmission are illustrated in Figure 3. In the literature, three different routes of infection with MAP are described. The main infectious route is the oral route. Although MAP is excreted through the faeces, milk and semen by infected animals (Sweeney, 1996), MAP faecal-contaminated colostrum, milk, or feed represents the primary sources of infection for susceptible hosts. Furthermore, MAP is also widespread in environmental samples of beef and/or dairy farms, such as in water (Norby et al., 2007; Raizman et al., 2004), slurry (Gwozdz, 2006; Jorgensen, 1977), sewage (Gwozdz, 2006) and sediment (Whittington et al., 2005). MAP is commonly found in environmental samples from the parlour exits, floors of holding pens, common alleyways, lagoons, manure spreaders and manure pits (Lombard et al., 2006; Raizman et al., 2004). The grass and soil of pastures can also be contaminated with MAP by the faecal excretion of grazing animals (Fecteau et al., 2010).



**Figure 3:** Theoretical MAP transmission pathways

The biggest problem for the control of MAP spread are the subclinically infected adult MAP shedders. These animals show no clinical signs, but excrete the pathogen intermittently in infectious concentrations (Münster et al., 2013b). In general, infected adult animals can be divided into three categories based on the degree of MAP shedding via the faeces (Raizman et al., 2007; Whitlock et al., 2000):

Low shedders: <10 CFU/tube

Moderate shedders: 10-49 CFU/tube

Heavy shedders: >50 CFU/tube.

Calves can also excrete MAP in their faeces; however, this potential source of infection is often underestimated because many studies focus on the examination of adult animals alone. Furthermore, it is difficult to identify infected calves because MAP is only excreted intermittently and in amounts below the limits of detection of the most-used diagnostic tool. In a recent study, the presence of MAP was demonstrated in the faeces of a naturally infected five-month-old calf (Hasonova et al., 2009). It has also been demonstrated in a longitudinal study of experimentally infected calves that a peak of MAP shedding occurs shortly after MAP inoculation and again two months after inoculation (Mortier et al., 2014).

Other sources of infection are milk and colostrum from subclinically and clinically infected cows. In the literature, pathogen concentrations of 2-8 CFU per 50 ml milk were reported in the milk of infected dairy cattle (Sweeney et al., 1992a). However, the mechanism of MAP extraction into milk remains unclear. An explanation for this may be that MAP is directly excreted into milk from lymphatics draining into the mammary glands. Indirect faecal contamination of the milk during the milking process is also likely (Hasonova et al., 2009).

MAP is systemically spread in infected hosts. Therefore, prenatal transmission of the pathogen from clinically and subclinically infected cows to their foetuses is common and well recognized (Seitz et al., 1989; Sweeney et al., 1992b). A meta-analysis estimated that the prevalence of prenatal infection is 9% (95% confidence interval [CI]: 6-14%) for subclinically diseased cows and 39% (95% CI: 20-60%) for clinically diseased cows (Whittington and Windsor, 2009). However, it is currently unknown

whether such intrauterine infected animals more rapidly develop clinical disease and what their shedding or antibody response pattern is.

Transmission of MAP via semen may be possible; however, this route of transmission is unlikely as in most previous studies only low concentrations of MAP were measurable in the ejaculate from infected bulls (Buergelt et al., 2004; Philpott, 1993). Furthermore, the transmission of MAP via the semen to a healthy cow has never been reported. However, Merkai et al. (1982) demonstrated that MAP can be found in cow uterine fluids and uterine walls after artificial contamination of the uterus. A longitudinal study of a subclinically infected bull showed that MAP is repeatedly detectable in semen in concentrations of up to  $10^5$  MAP genome equivalent/ml semen. MAP has also been found in the male reproductive organs (Table 6). A prevalence study showed that 20.0% of the breeding (47/235) and 33.3% of the teaser bulls (6/18) semen production units in Punjab (Pakistan) had a serological antibody response against MAP (Abbas et al., 2011). It has also been known for over 45 years that MAP could survive the antibiotic treatment and freezing procedure of yore used in artificial insemination (Larsen and Kopecky, 1970). Therefore, bulls should be considered a potential source of transmission, especially due to their direct and indirect (artificial insemination) contact with many cows (Khol et al., 2010).

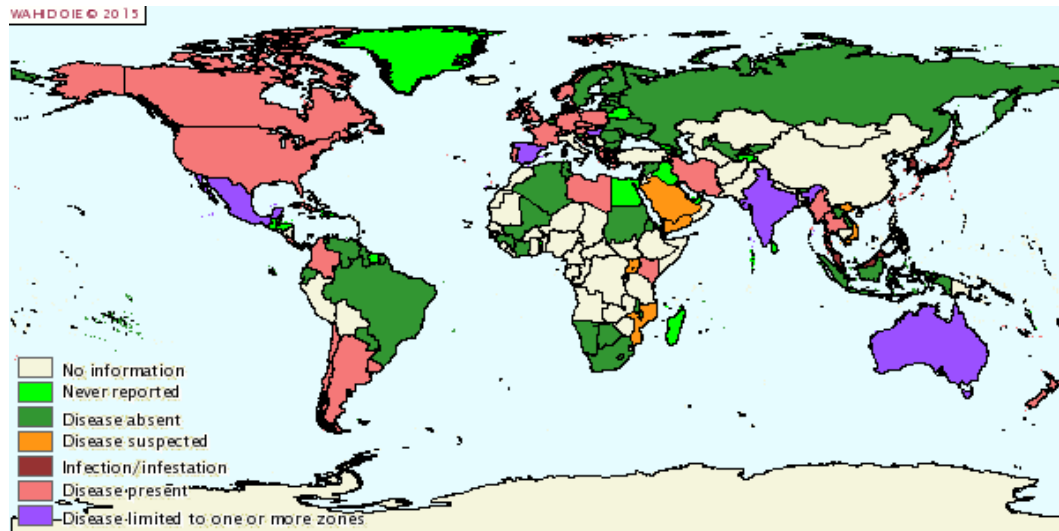
Another source of infection for ruminates appears to be MAP-containing dust. Under experimental and natural conditions, the presence of MAP in settled dust collected from dairy farms was confirmed (Eisenberg et al., 2010a; Eisenberg et al., 2010b). Furthermore, recent experimental studies have demonstrated that an infection with MAP in cattle as well as in sheep could occur by the inhalation of MAP through the nasal and transtracheal routes. An infection via both routes can trigger a manifestation of MAP in the intestinal tract and corresponding lymph nodes.

The introduction of MAP into former paratuberculosis-free herds most likely occurs by the purchase of diagnostically and clinically unrecognized infected animals (Sweeney, 1996). Another source of MAP introduction may be manure movement between farms, the purchase of colostrum or milk and the sharing of pastures or water between farms (Lombard, 2011). In addition to the transmission between domestic ruminants, wild ruminants, and a wide range of non-ruminants can also be infected with MAP. Wild ruminant species such as the white-tailed deer (*Odocoileus virginianus*) (Chiodini and Van Kruiningen, 1983), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*)

(Nebbia et al., 2000; Robino et al., 2008) and fallow deer (*Cervus dama dama*) (Balseiro et al., 2008) seem to be true MAP reservoirs (Carta et al., 2013). In contrast to such diseased ruminant species, infected non-ruminants usually do not develop macroscopic or microscopic lesions. The only well-studied non-ruminant species are wild rabbits. These animals can excrete MAP in concentrations of up to  $7.6 \times 10^5$  CFU/g in faeces (Daniels et al., 2003). Furthermore, MAP has also been detected in the urine of MAP-infected rabbits (Daniels et al., 2003). It was recently demonstrated that the high prevalence of MAP in rabbits is associated with difficulties in controlling paratuberculosis in cattle farms in East Scotland (Shaughnessy et al., 2013). Compared with this knowledge about rabbits, only a few case reports on other clinically diseased non-ruminant species have been described in the literature. One example of this is a MAP-infected miniature donkey (*Equus asinus f. asinus*) with typical clinical and pathomorphological changes (Stief et al., 2012). Transmission between wild and domestic animals most likely occurs via the intake of MAP faecal-contaminated material due to the shared use of the same environment, such as pasture area (Carta et al., 2013).

## PREVALENCE

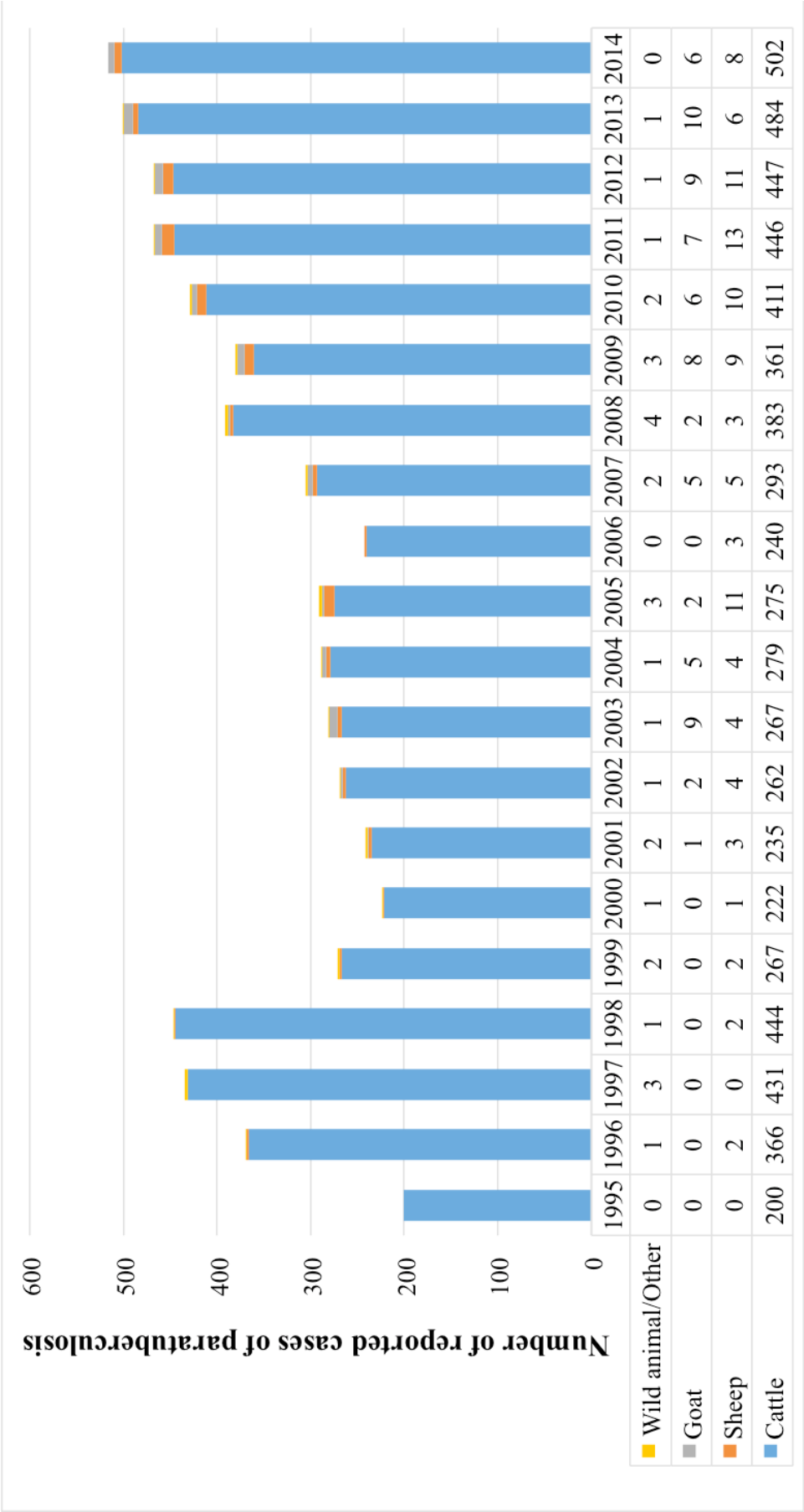
Paratuberculosis is distributed worldwide (Turenne and Alexander, 2010) (Figure 4). The World Organisation for Animal Health (OIE) classifies paratuberculosis as a disease with socioeconomic importance and significance for human health. Several studies have revealed a herd level prevalence of 6-55% in Europe (Boelaert et al., 2000; Gasteiner et al., 1999; Manning and Collins, 2001); however, only a few studies have provided comparable and reliable estimates of the prevalence of MAP infection in different ruminants. The following grounds are the main reasons for the lack of comparable prevalence: i) Varying definitions of the condition “paratuberculosis,” ii) difficulties in detecting the condition using the currently available diagnostic options, and iii) deficient study designs. Based on these aspects, a review analysing the published herd and animal prevalence of MAP infection in farmed animals in Europe was highly critical of the findings. A proper conclusion regarding the prevalence of MAP infections in Europe cannot be made. Estimates of the true prevalence among cattle are approximately 20%, and herd level prevalence is likely to be >50% in many countries (Nielsen and Toft, 2009).



**Figure 4:** Worldwide presence of paratuberculosis in 2015 (OIE, 06.11.2015)

Paratuberculosis is also widespread throughout Germany, and is a notifiable disease. In Germany, all confirmed cases of paratuberculosis are documented in the animal disease intelligence database. In 2014, the number of confirmed paratuberculosis cases had increasing to 502 cases per year. In Figure 5, the reported cases of paratuberculosis between 1995 and 2014 are listed in detail. In Lower Saxony, a voluntary control program was introduced in 1990. Paratuberculosis control programs have also been established in other federal states, such as North Rhine-Westphalia and Thuringia. No comprehensive, nationwide studies on the prevalence of paratuberculosis have been performed. Therefore, an estimation of disease prevalence in Germany is currently not possible.





**Figure 5:** Number of reported cases of paratuberculosis by the animal diseases intelligence service in Germany between 1995 and 2014

## HOST RANGE

In principle, paratuberculosis is a diarrhoeal disease of domestic ruminants. Bovine, ovine and caprine animals are particularly affected. The causative agent is also found in a large number of cervids and other zoo and wild ruminants, such as bison, antelope, camelid, zebu, yak, and water buffalo (Chiodini et al., 1984a; Deutz et al., 2005; Godfroid et al., 2005; Manning et al., 2003; Ridge et al., 1995; Sivakumar et al., 2005; Zavgorodniy et al., 2015). The typical symptoms of the disease can also develop in some wild and zoo ruminants (Figure 6). Infected wild animals likely play an important role in the spread of MAP, forming a potential source of infection for domestic ruminants (Chiodini and Van Kruiningen, 1983).



**Figure 6:** Alpaca in the final stage of paratuberculosis (veterinarian Aloys von Buchholz, Rheine)

The pathogen has also been detected in monogastric animals. In most cases, these infected animals do not develop clinical symptoms (Chiodini et al., 1984a). Monogastric animals, in which the pathogen can be isolated, include for example non-human primates (McClure et al., 1987; Singh et al., 2011; Zwick et al., 2002), foxes (Beard et al., 1999), snow bunnies (Deutz et al., 2005), raccoons and banded animals (Corn et al., 2005). In the case of wild rabbits, the pathogen has been isolated from faeces and pathological changes were found during autopsy that were very similar to

those expected in paratuberculosis (Greig et al., 1997). McClure et al. (1987) performed investigations in a stump-tail macaque herd. Samples from a total of 38 animals were analysed using PCR. MAP was detected in 28 animals, and 13 of these animals died within five years. The deceased animals showed typical symptoms of paratuberculosis.

#### ZOONOTIC ASPECT

For the first time, Dalziel (1913) associated the chronic inflammation of the human intestine known as Crohn's disease (CD) with the intestinal infection of cattle known as paratuberculosis. Since that time, the relationship between MAP and CD has been debated. Nevertheless, the “Guidelines for the management of paratuberculosis in ruminants”, published by the German Federal Ministry of Food, Agriculture and Consumer Protection (Anonymous, 2005), advised the potential risk for humans to get infected with MAP. MAP is widespread in the food chain. Humans may be exposed to MAP through the consumption of dairy and beef products such as meat (Alonso-Hearn et al., 2009), milk (Ayele et al., 2005; Ellingson et al., 2005; Grant et al., 2002) and cheese (Spahr and Schafroth, 2001).

MAP was initially isolated from three CD patients in 1984 (Chiodini et al., 1984b). Since then, the different matrices of CD patients have been tested for MAP in many other studies (Kirkwood et al., 2009; Schwartz et al., 2000). For instance, in one study, the intestinal mucosal tissue of Sardinian CD patients and non-diseased people, who served as controls, were investigated for the presence of MAP using PCR (Sechi et al., 2005). The pathogen was detected in 25 patients (83.3%) with CD and three control patients (10.3%). To confirm the PCR results, tissues were also prepared for culture. Tissue culture confirmed the presence of MAP in all PCR-positive cases. In another study, MAP DNA was detected via PCR in blood samples from 13 (46%) patients with CD, four (45%) patients with ulcerative colitis (UC) and three (20%) patients without inflammatory bowel disease. In that study, viable MAP was cultured from the blood of 14 (50%) patients with CD and from two (22%) patients with UC (Naser et al., 2004). Such studies indicate that MAP may be involved in the pathogenesis of CD. However, in other studies, MAP was not detected in the tissues of CD patients. For instance, MAP DNA was not present in intestinal biopsies of 81 Indian patients with CD (Sasikala et al., 2009).

The aetiology of CD remains largely unknown. An infectious cause, a genetic predisposition, and an auto-immune-mediated phenomenon may all be involved in the pathogenesis of CD (Büttner et al., 2006).

### CONTROL MEASURES IN EUROPE

The occurrence of paratuberculosis is not underestimated only in Germany. There are indications that the infection is endemic in most countries with a developed dairy industry (Kennedy and Benedictus, 2001). Similarly, a high prevalence is likely in most countries with cattle farming, although accurate estimations are not available (Nielsen and Toft, 2009). Control and monitoring programs are already underway in some countries (Bakker, 2010).

In the Netherlands, the aim of the program is not the certification of MAP-free herds, but the reduce of MAP concentration in milk. The Dutch Animal Health Service launched its quality assurance program in 2006. This program is based on the annual testing of milk samples from all dairy cattle and serum from all other cattle older than three years via ELISA. On the basis of the test results, the herds are classified into status A (negative test results), status B (positive result, corresponding animal was removed) or status C (positive result, corresponding animal remains in the herd) (Weber and Schaik, 2008). Since 2010, participation in the program has been mandatory. In order to ensure the quality of milk, since the first of January 2011 only the milk from herds with status A or B can be used by the dairy industry.

In Luxembourg, all animals older than 24 months have been tested using ELISA. The classification into different statuses is based on the percentage of test-positive animals within a herd. A herd with status A has 0% ELISA-positive animals, status B has <5%, status C 5-10%, and status D >10% ELISA-positive animals. Herds without sampling or without removal of positive animals are marked as level O. Based on the status of a herd, different measurements are then recommended (Bakker, 2010).

In Denmark, a voluntary monitoring program has existed since 2006. It aims to provide tools for the farmer to control MAP infection and to reduce the prevalence within the country (Nielsen, 2007). Part of this program is the elevation of the paratuberculosis status based on the results of a three-monthly milk ELISA. In contrast to other programs, the herds are not classified into different categories.

In Belgium, all animals older than 30 months are tested, via a milk ELISA for dairy cattle and serum ELISA for dry cattle. Cattle that test positive are slaughtered within six months. This voluntary control program has existed since 2008.

In France, two national control programs exist: one to control herds that are infected with MAP and one to monitor MAP-free herds. The test regime depends on the status of the herd (one recently infected head of cattle or herds with endemic paratuberculosis). In herds with endemic paratuberculosis, faecal culture or PCR is recommended. Farmers are allowed to leave the program under certain conditions, for example if all tests have been negative in the previous two years. For the monitoring of MAP-free herds, animals that are older than 24 months are tested at an interval of between nine and 30 months by ELISA, faecal culture or PCR (Bakker, 2010).

In Austria, a compulsory control program for clinical paratuberculosis in sheep, goats and farmed deer has existed since 2006. In Italy and Spain, there is currently no nationwide established paratuberculosis control.

In Germany, no nationwide control program currently exists. Several federal states have established voluntary control programs, for example North Rhine-Westphalia (since 1998), Saxony (2003), Thuringia (2008), Hessen (2014), and Rhineland Palatinate. Guidelines for the control of paratuberculosis were published by the government in the “Recommendations of the Federal Ministry of Food and Agriculture on hygienic requirements for the keeping of ruminants” (Bätza, 2014). The aims of these guidelines are the standardisation of control measures in Germany, the prevention of MAP spread, the minimization of clinical cases and economical losses introduced by MAP, and prevalence reduction. On this basis, Thuringia published a new program for the control of paratuberculosis (Anonymous, 2015). The aims of this program are i) to reduce the prevalence of MAP infections in the herds, ii) contain spread to other cattle herds, and iii) the creation and protection of paratuberculosis-free stocks. In this program, three different phase of control were defined.

### *1. Control phase*

This phase is divided into four different levels:

Level 1: For the evaluation of the herd status, semi-annual environmental faecal samples or pooled faecal samples are examined using bacteriological tests.

Level 2: Heavy shedders (high risk animals) are identified annually by serological examination of all cattle older than 24 months. Animals testing positive should be slaughtered as soon as possible.

Level 3: For the identification of subclinically infected animals, faeces are annually examined using bacteriological methods. All animals that return a positive test should be slaughtered as soon as possible.

Level 4: In addition to all measures found in Level 3, cattle testing positive must be killed within one month after detection, and pregnant cattle must be killed within one month after calving.

2. *Recognition and acquisition phase of the status "paratuberculosis unsuspected herd"*

A herd is regarded as unsuspicious for paratuberculosis when the results of the examination of the recognition phase have been negative over a continuous period of three years, and when the obligatory hygienic measures have been completed. During the period of recognition, only cattle from herds of the same or higher phase and level of control should be purchased.

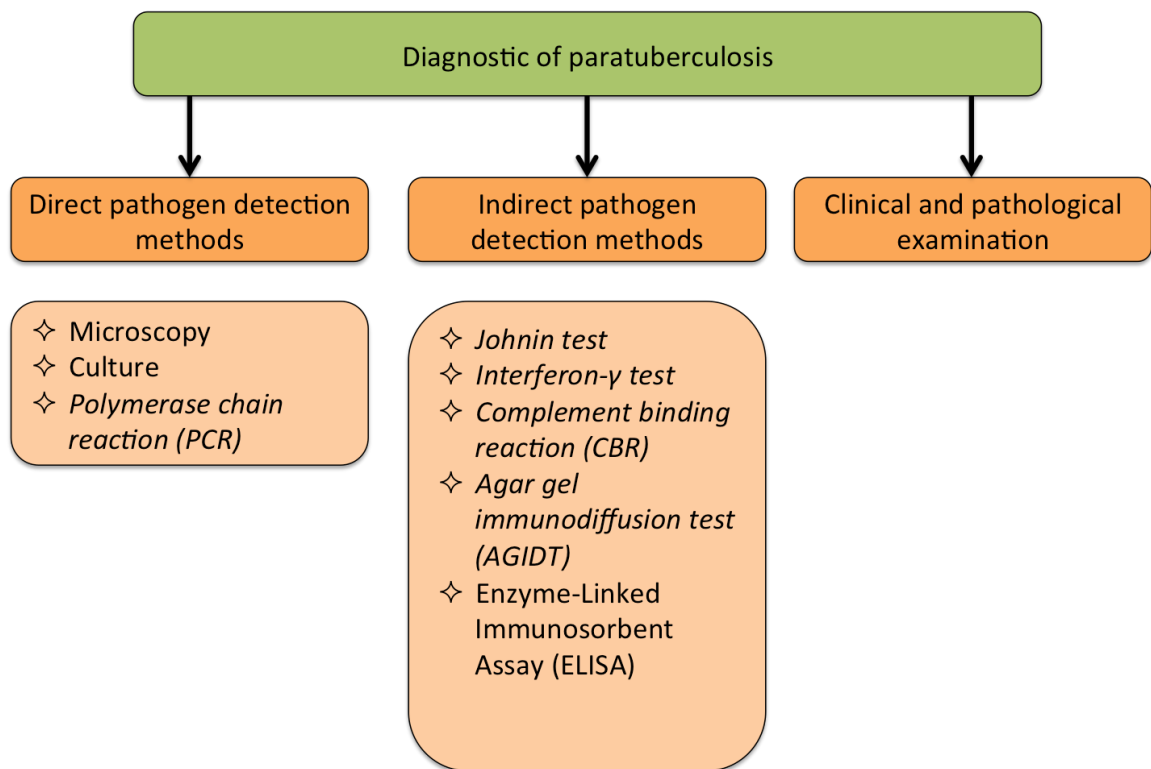
3. *Monitoring phase*

In order to maintain the status, cattle older than 30 months are examined using faecal testing at an interval of two years. In addition, environmental faecal samples are investigated for the presence of MAP on a semi-annual basis. Furthermore, animals with typical signs of disease are to be immediately examined using bacteriological methods. The obligatory hygienic measures must also be completed.

The hygienic measures of the program have the following objectives: i) Prevention of the new infection of young animals via the pathogen-free rearing of calves, ii) reduction in infection pressure by the early detection and removal of infected animals from the herd, and iii) prevention of disease introduction by the careful purchase and access of animals.

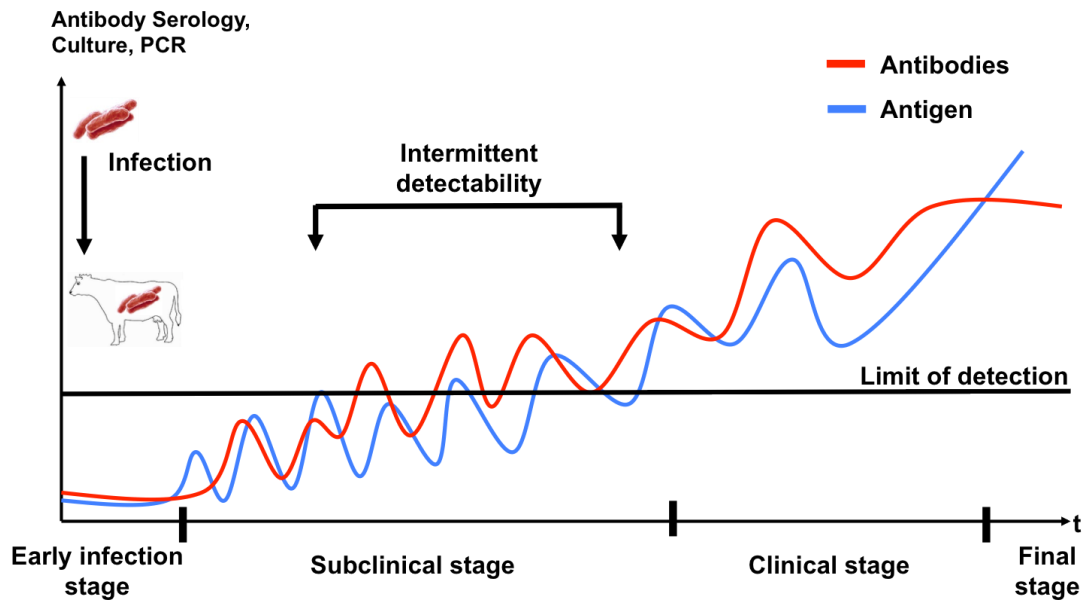
## DIAGNOSTIC METHODS

Ante-mortem diagnostic methods for MAP can be grouped into tests measuring the cell-mediated or humoral immune response against MAP, and tests detecting the pathogen (Figure 7).



**Figure 7:** Diagnostic options to detect a MAP infection

Depending on the disease stage, the sensitivity and specificity of diagnostic tests vary (Nielsen and Toft, 2009). Due to the chronic character of paratuberculosis infection, the age of the animals influences the stage of the disease and hence the test response (Figure 8).



**Figure 8:** Detectability of the MAP agent or antibody response during different disease stages

## INDIRECT DIAGNOSTIC METHODS

MAP-specific antibodies or a host MAP-specific cellular immune response are detected using indirect methods. A differentiation is here made between allergological (cell-based) and serological (antibody-based) methods.

### A) *Johnin test*

The Johnin test is based on the cellular immune response, and belongs to the allergological test methods. For detection of the immune response, an avian tuberculin or Johnin/paratuberculin solution is administered to the animal either intradermally or intravenously. In the case of intradermal application, evaluation is made by measuring the thickness of the skin fold at the injection site. A differentiation is made between two reaction types (Merkal, 1973). The immediate type is an allergic reaction. This reaction is unspecific and manifests within two hours by an oedematous swelling at the injection site. Reactions occurring after 24 to 72 hours are the delayed reaction type. This results in a profound increase in wheal circumference at the injection site. In the intravenous application of the solution, the evaluation is based on the body temperature profile or the ratio of neutrophil granulocytes to lymphocytes (Chiodini et al., 1984a). However, it



must be noted that cross-reactions with other mycobacteria can occur that distort the results (Clarke, 1997).

#### *B) Interferon- $\gamma$ test*

Another method based on the cellular (T cell-driven) immune response is the interferon- $\gamma$  test. The mechanism of this test is that the T cells present in the blood are stimulated by mycobacterial antigens, producing interferon- $\gamma$ . This cytokine is detectable using IFN- $\gamma$  ELISA (Stabel, 1996). An advantage of this method is that even young, infected animals with a low humoral immune response can be identified, and thus the test is also suitable for the detection of the disease in the early stages (Stabel et al., 2007). However, the sensitivity is limited to values between 13 and 85%, and the specificity is moderate, with values between 66 and 67%. Based on these parameters, the test is currently unsuitable for paratuberculosis diagnostics (Büttner et al., 2006).

#### *C) Complement-binding reaction (CBR)*

CBR was the first serological test designed for the detection of MAP antibodies in blood serum. The CBR is based on the detection of complement-binding antibodies in the serum of tested cattle. This test was previously obligatory for the international trade of cattle; however, the test lacks sensitivity, especially during the subclinical stage of disease. The possibility of cross-reactivity also had a limiting effect on specificity (Wilks et al., 1981). For these reasons, it is no longer used today (Sherman et al., 1990).

#### *D) Agar gel immunodiffusion test (AGIDT)*

The advantages of the AGIDT are that it is both cost-effective and fast. In this assay, MAP antigens are located in a gel. The tested serum is applied to the gel. Detection of MAP antibodies is via the antigen-antibody binding. The sensitivity is limited to values between 8.3 and 29.1% (Ferreira et al., 2002; Gumber et al., 2006; Robbe-Austerman et al., 2006). The specificity of the method is between 92.5 and 99.5% (Ferreira et al., 2002; Robbe-Austerman et al., 2006). The AGIDT is particularly suitable for the rapid diagnosis of clinically ill animals, since these have a correspondingly high antibody level. Only at this stage does the test have a sufficient sensitivity (more than 50%). However, even in clinically infected animals with progressive infection, often no antibodies are detected.

### E) Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA assays have the highest diagnostic value in the serological methods, since the antibody level has already increased prior to the occurrence of clinical signs. Although ELISA tests are currently well-suited to herd diagnostics, they have limitations in the diagnosis of individual animals. For paratuberculosis diagnostics, MAP-specific antigens are usually bound to a microtiter plate. Milk or serum can thus be tested for the presence of antibodies. When specific antibodies to MAP are present, an antigen-antibody complex is formed in the respective well. An enzyme-linked anti-ruminant immunoglobulin antibody is added to the complex, along with a chromogenic substrate, which then shows a photometrically measurable colour change.

The ELISA technique was initially in 1971 (Engvall and Perlmann). Seven years later, this method was used for the diagnosis of paratuberculosis (Berg and Jensen, 1978). Thereafter, further ELISA tests were developed with different but a significantly higher sensitivity and specificity than in CBR and AGIDT (Reichel et al., 1999). At present, a total of four manufacturers in Germany offer certified ELISA assays for the diagnosis of paratuberculosis. For the coating of the microtiter plates, for example, protoplasmic antigens or cell wall components are used in these ELISA methods. Studies have shown that the specificity is less pronounced without pre-absorption using *Mycobacterium phlei*. Cross-reactivity is significantly reduced by sera pre-absorption (Köhler et al., 2007). Furthermore, the sensitivity of the respective ELISA method depends on the stage of the infection. In subclinically infected animals, lower diagnostic sensitivities are generally found than in animals in the advanced disease stage. The diagnostic sensitivity of the tests in weak shedders is between 15.0 and 26.0%, and in strong shedders between 75.9 and 88.1% (Dargatz et al., 2001; Köhler et al., 2007; Sweeney et al., 2006; Whitlock et al., 2000).

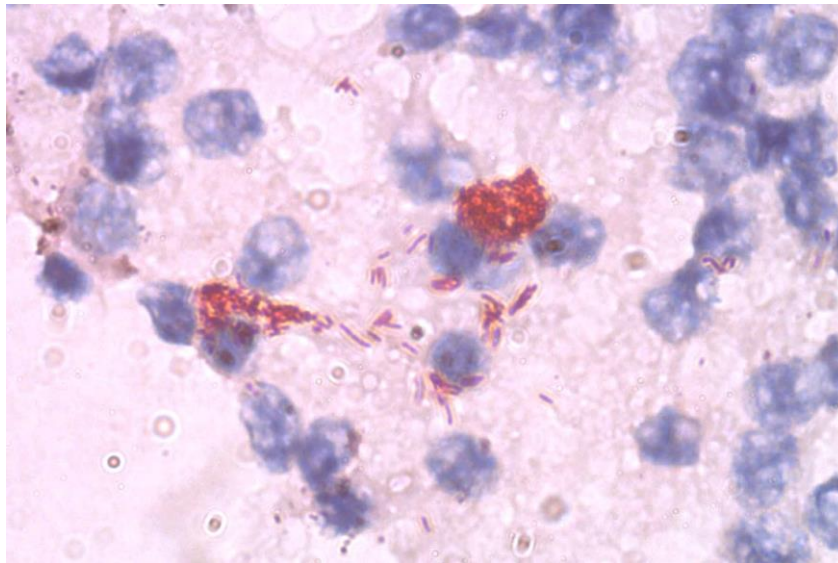
## DIRECT DIAGNOSTIC METHODS

In the case of direct pathogen detection, the pathogen or its DNA is detected.

### A) Microscopy

The direct microscopic detection of pathogens by Ziehl-Neelsen staining is a cost-effective and simple method to carry out (Figure 9). Ziehl-Neelsen staining is based on

the acid and alcohol stability of MAP and can be derived from many different matrices, for example faeces, rectal mucosa or mesenteric lymph nodes. Due to the particular cell wall properties of MAP, the red dye carbol fuchsin cannot be removed by treatment with hydrochloric alcohol. Using the light microscope, MAP bacteria are marked as red-coloured, slender rods, which form clusters via intracellular filaments (Merkal, 1973). However, caution must be taken when interpreting the results, as this method has a low analytical sensitivity and specificity (Benazzi et al., 1996).



**Figure 9:** Visualization of MAP in lymphatic tissue using Ziehl-Neelsen staining (Division of Microbiology and Animal Hygiene, Georg-August-University Göttingen)

#### *B) Cultural detection of MAP*

Cultural evidence from faeces and organ samples is still regarded as the "gold standard" for diagnosis (Köhler and Burkert, 2003). However, detection is difficult due to the slow growth of MAP (Lambrecht et al., 1988; Whipple et al., 1991). Various media such as the Herrold's Egg Yolk Medium (HEYM; Figure 10) and liquid culture media (Mycobacterial Growth Indicator Tube [MGIT]) are currently available for bacterial culture (Hughes et al., 2001). A further problem encountered in culturing is decontamination (Stabel, 1996). In order to be able to detect the slowly-growing pathogen, accompanying flora such as bacteria and fungi must be inhibited by the addition of for example Hexadecylpyridinium chloride (HPC) (Collins, 1996). A positive MAP culture is considered to be 100% specific when a subsequent hedging by

for example PCR is carried out. The diagnostic sensitivity is strongly dependent on the disease stage of the animal, and is approximately 50% (Sherman et al., 1984; Sockett et al., 1992; Stabel, 1998). Further factors influencing the sensitivity of the test are the pathogen concentration in the sample, the storage and processing conditions, and the cultivation method used (Eamens et al., 2000).



**Figure 10:** Growth of MAP colonies on HEYM (Division of Microbiology and Animal Hygiene, Georg-August-University Göttingen)

### *C) Polymerase chain reaction (PCR)*

Another direct detection method is PCR (polymerase chain reaction). PCR is fast and simple to perform, and a high sample throughput is possible (Köhler, 2005). MAP DNA can be detected from faeces (Whipple et al., 1992), blood (Koenig et al., 1993), various organs (Englund et al., 2001) and lymph nodes (Challans et al., 1994) using PCR.

Several different PCR methods have been developed for the detection of MAP. In the nested and semi-nested PCR, two PCR assays are connected in series. The product of the first PCR run is used as the template for the subsequent PCR run. In this way, it is possible to detect very small amounts of DNA. However, the reaction vessels must be

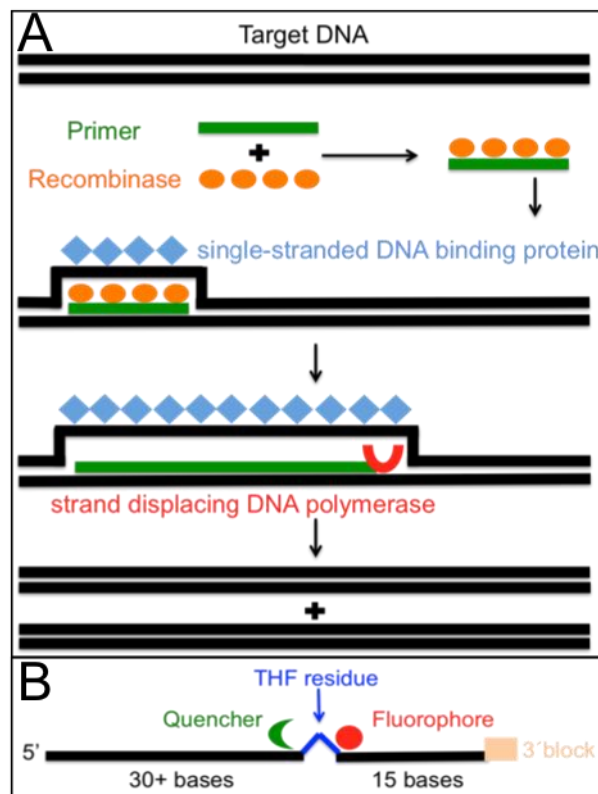
opened after the first PCR run, increasing the risk of contamination. This risk is significantly lower when using real-time PCR; the workload and time required are also reduced. Real-time PCR is an automated method in which quantification is performed by fluorescence measurement during a PCR cycle.

Most PCRs are based on the MAP-specific insertion sequence IS900, which was discovered by Green et al. (1989). This sequence is 1453 bp in length and is present in 17 largely identical copies within the MAP genome (Li et al., 2005). Analysis of bovine faecal samples revealed diagnostic sensitivities of 54%, 49%, 48% and a specificity of 100% using three different PCR methods, which were based on the IS900 genes (Taddei et al., 2004). Other MAP-specific insertion sequences are ISMav2 (Strommenger et al., 2001), ISMap02 (Stabel and Bannantine, 2005), the hspX gene (Ellingson et al., 1998), ISMpa1 (Olsen et al., 2004), IS\_MAP04 (Li et al., 2005) and the F57 sequence (Vansnick et al., 2004).

#### *D) Isothermal amplification*

Methods of DNA amplification at a constant temperature are called isothermal amplification (Li and Macdonald, 2015). Many different technologies to accomplish this have been established in the last 20 years, for example loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000), nicking enzyme amplification reaction (Van Ness et al., 2003), rolling circle amplification methods (Gusev et al., 2001), helicase dependent amplification (Vincent et al., 2004) and recombinase polymerase amplification (RPA) (Piepenburg et al., 2006). LAMP is the most widely used method (Cook et al., 2015; Hopkins et al., 2013; Morris et al., 2015; Polley et al., 2013). LAMP is performed at 60 °C, and the results are obtained after 50-60 minutes. However, LAMP utilizes six primers, which are difficult to design, especially for highly variable pathogens. The LAMP method does not include a probe, which leads in some cases to nonspecific results. In contrast, RPA is extremely fast (three to ten minutes), runs at 42 °C and uses two primers and one exo-probe. Amplification in the RPA depends on the binding of the recombinase to the oligonucleotide primers (Figure 11A). The complex then scans the template DNA for the corresponding sequence and initiates 5'-strand invasion of the oligonucleotide at the site of homology. The strand invasion is stabilized by the single-strand binding protein interacting with the complementary DNA strand to avoid primer ejection. Primer extension ensues via a strand-displacing DNA polymerase (Piepenburg et al., 2006). Real-time detection of RPA amplicons is possible via exo-

probes (Euler et al., 2012a; Euler et al., 2012b) (Figure 11B). The development of fluorescence depends on the separation of fluorophore and quencher via Exonuclease III cleaving at an internal abasic site mimic (tetrahydrofuran, THF) of the hybridized exo-probe. The fluorescence signal is measured in real-time via a simple point-of-care scanner, weighing 1 kg, fixed in the mobile suitcase laboratory (Abd El Wahed et al., 2013a; Abd El Wahed et al., 2013b; Amer et al., 2013; Piepenburg et al., 2006).



**Figure 11:** Principle of RPA method. **A:** The amplification cycle of the RPA showing only the forward primer; **B:** The structure of the RPA exo-probe for the detection phase of the amplicon (Ahmed Abd El Wahed, Division of Microbiology and Animal Hygiene, University of Göttingen, Germany)

## SCOPE OF THIS THESIS

The major scope of this thesis is to investigate the unrecognized spread of MAP by asymptotically infected hosts. These animals do not necessarily exhibit clinical signs and a reliable diagnosis is not available. This leads to a challenge in ensuring efficient

disease control during the subclinical stage of the disease. Therefore, the occurrence of MAP in a clinically asymptomatic bull as well as different non-ruminant species is examined using different diagnostic methods. Furthermore, possible transmission pathways through the excretion of MAP within animals of the same species and between different species are examined.

Initially, the pattern of MAP occurrence in a naturally infected, but asymptomatic, bull is investigated in **chapter I**. In this chapter, the presence of MAP is monitored in faeces, blood and semen over a period of four years by qualitative and quantitative PCRs and bacterial culture. Antibody response is additionally measured by commercially available ELISA kits. After necropsy, the distribution of MAP within the subclinical bull is evaluated by PCR and bacterial culture. In **chapter II**, the susceptibility of non-human primates to MAP 20 animals (belonging to 7 different species) is investigated. After necropsy, faecal and tissue samples are analysed via qualitative and quantitative PCRs and bacterial culture. Only little information are available on the distribution of MAP among wildlife, therefore, **chapter III** addresses the occurrence of MAP in a group of rock hyraxes. Animals were once captured in South Africa and kept in quarantine in a German zoological garden. Finally, in **chapter IV**, a recombinase polymerase amplification (RPA) assay is developed for the fast detection of MAP, especially at the point of need.

## **2 STUDIES PERFORMED**



CHAPTER I

Paper published

*Transboundary and Emerging Diseases*

**Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in a Subclinical Naturally Infected German Fleckvieh Bull**

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## Summary

Although it has been known for years that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is detectable in the reproductive organs and semen of infected bulls, only few studies have been conducted on this topic worldwide. This study surveyed the MAP status of a bull, naturally infected due to close contact with its subclinically infected parents over a period of 4 years. From the age of 7 weeks to necropsy, faecal, blood and, after sexual maturity, semen samples were drawn repeatedly. Already at the first sampling day, MAP-DNA was detected in faeces by semi-nested PCR. True infection was confirmed by the detection of MAP-DNA in blood at the age of 40 weeks. In total, MAP-DNA was present in 25% faecal (34/ 139), 16% blood (23/140) and 5% semen (4/89) samples, including MAP-free intervals of up to 9 weeks. MAP genome equivalents (MAP-GE) of up to  $6.3 \times 10^6$ /g faeces and  $1.8 \times 10^5$ /ml blood were determined. Cultivation of MAP occurred only in three of 137 faecal and two of 109 blood, but never in semen samples. Over the whole period, the bull was a serological negative MAP shedder. During necropsy, 42 tissue samples were collected. Neither macroscopic nor histological lesions characteristic of a MAP infection were observed. Cultivation of MAP in tissue sections failed. However, MAP-DNA was spread widely in the host, including in tissues of the lymphatic system (7/15), digestive tract (5/14) and the urogenital tract (5/9) with concentrations of up to  $3.9 \times 10^6$  MAP-GE/g tissue. The study highlighted the detection of MAP in male reproductive organs and semen. It supports the hypothesis that bulls may probably transmit MAP, at least under natural mating conditions. In artificial insemination, this might not be relevant, due to antibiotics included currently in semen extenders. However, the survivability of MAP in this microenvironment should be investigated in detail.

**Keywords:** *Mycobacterium avium* subsp. *paratuberculosis*; pathogenesis; distribution pattern; PCR; German Fleckvieh bull; subclinical infection

## Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease, which mainly infects domestic ruminants (cattle, sheep and goats). It is characterized by a degenerative chronic granulomatous inflammation of the digestive tract. The resulting profuse diarrhoea, emaciation and progressive weight loss have a considerable economic impact on the dairy and beef industry (Raizman et al., 2009).

In general, an important part in the transmission of MAP is the infection of the calf. Newborn calves are mostly affected by the faecal–oral route; colostrum and milk from subclinically (Streeter et al., 1995) or clinically infected cows (Taylor et al., 1981) are further sources for infection. Another possible pathway of the infection represents the airborne transmission of MAP by contaminated bioaerosols (Eisenberg et al., 2011). The role of bulls in the transmission of MAP via semen is still unclear even though it has been known since 1970 that MAP is detectable in the semen and reproductive organs (Larsen and Kopecky, 1970). To our knowledge, the venereal transmission of MAP by semen was never demonstrated in the past. As opposed to this, it is generally accepted that MAP is transmitted vertically from the cow to their foetus in utero (Pribylova et al., 2013). A meta-analysis indicated that the total prevalence of calves infected in utero was 13% among infected cattle (Whittington and Windsor, 2009). In cows, the pathogen was found in uterus tissue and uterine flush, as well as in foetuses (Lambeth et al., 2004; Hasonova et al., 2009). In bulls with (Larsen and Kopecky, 1970; Larsen et al., 1981; Glawischnig et al., 2004; Khol et al., 2010) and without clinical signs (Ayele et al., 2004; Münster et al., 2013), MAP was detected in the testicle, epididymis, seminal gland, prostate gland and bulbourethral gland as well as their semen. In one of the investigated, asymptomatic bulls (Münster et al., 2013), MAP was intermittently detected (44.6%; 29/65) in concentrations of up to  $5.7 \times 10^5$  MAP genome equivalents/ml semen over a period of 54 months. Although it has been demonstrated previously that bulls excrete the pathogen via semen, the relevance of breeding bulls in the distribution of MAP, especially when they are in the subclinical stage of the disease, is still unknown. In the past, it has been shown that MAP survived the freezing process and antibiotic treatment customarily used for commercial semen samples at that time (Larsen and Kopecky, 1970).

The aim of this study was to characterize the distribution of MAP in faecal, blood and semen samples of an asymptomatic German Fleckvieh bull, which was naturally

infected by its subclinically infected parents, over the period of 4 years from the age of 7 weeks to necropsy. The analysis of tissue samples, taken during necropsy, provides a detailed overview of the MAP distribution within a subclinical male bovine host.

## **Materials and Methods**

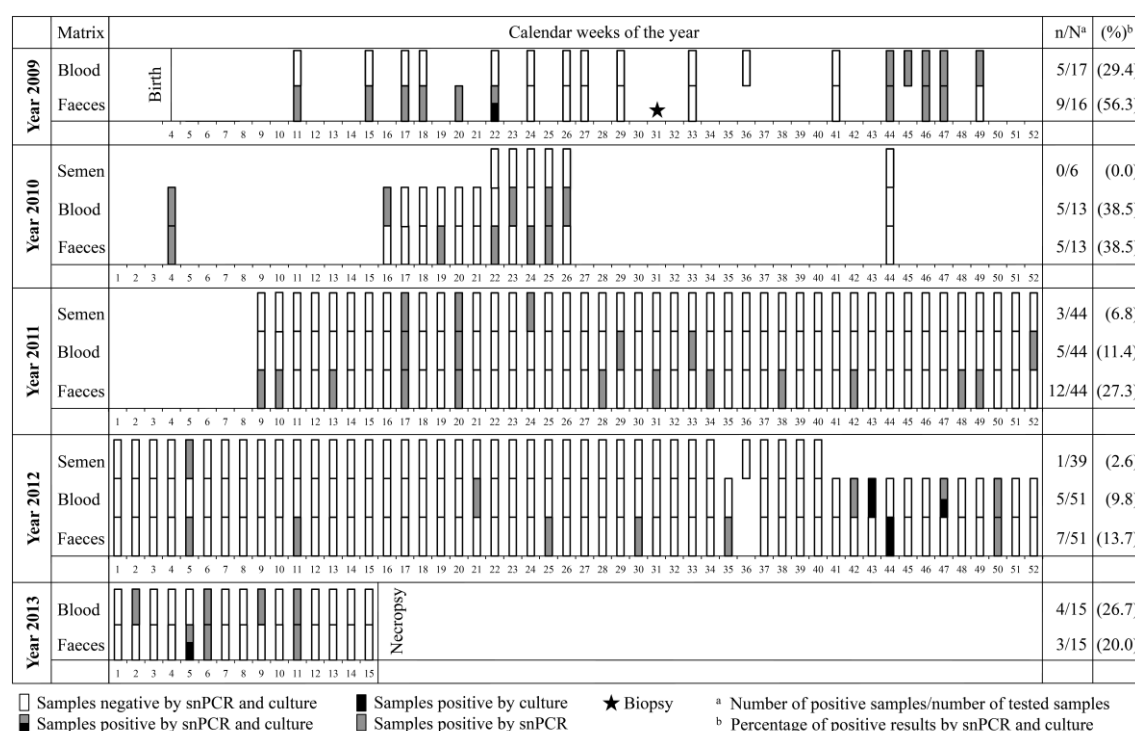
### ***Animal***

The German Fleckvieh bull (related to Simmental; *Bos primigenius taurus*) was born on 29 January 2009 in the 4th calendar week (CW) at the Institute of Veterinary Medicine (University of Göttingen, Germany). The infection progress of the bull's subclinically infected father was published already (Münster et al., 2013). The subclinically infected mother tested positive for MAP in blood and faeces during routine controls, but an antibody response was not measured (data not shown). During the suckling period of the bull calf, the parents were tethered and the calf could move freely in a conventional stable. After the suckling period, the bull calf was isolated in quarantine. Feeding and environmental conditions were maintained. By routine diagnostic testing, infections with BHV1, BVD, BT, brucellosis, leucosis, leptospirosis and Q-fever were excluded.

### ***Longitudinal study***

From the age of 7 weeks to necropsy (CW 11 in 2009 to CW 15 in 2013), samples were taken on 142 different dates (Fig. 1). In the first 2 years (CW 11 in 2009 to CW eight in 2011), samples were taken at irregular intervals from 1 to 17 weeks. Until CW 21 in 2010, blood and faeces were collected in parallel, with the exception of 3 days (CW 20, CW 36 and CW 45 in 2009). Once the bull was sexually mature (CW 22 in 2010), semen samples were also drawn repeatedly. From CW 9 in 2011, the study was extended and sampling from all three matrices occurred on a weekly basis. From CW 41 in 2012 until the end of the study, no more semen samples could be collected because the bull was showing aggressive behaviour towards the animal caregivers. In total, there were blood samples taken on 140 days, faecal samples on 139 days and semen samples on 89 days. To avoid faecal cross-contaminations, the order of sample collection was consistently the same; first semen, followed by blood withdrawal, and finally rectal faecal sampling. Prior to sampling, the bull and the teaser were sheared routinely and washed regularly including the genital area. For semen collection, the bull was mounted

on the teaser using a washed artificial vagina disinfected with 70% ethanol. Semen samples were collected in sterile tubes. Afterwards, blood was collected from the coccygeal vein. Skin was cleaned and disinfected with 70% ethanol before. Finally, faecal samples were taken directly from the rectum using sterile disposable gloves and transferred to sterile plastic bottles (250 ml). After collection, all samples were transported to the laboratory. Immediately, all collecting receptacles were cleaned externally and disinfected with 70% ethanol. Sample preparation was performed in the same order as mentioned for collection.



**Fig. 1.** Distribution patterns of *Mycobacterium avium* subsp. *paratuberculosis* in faeces, semen and blood of a naturally infected German Fleckvieh bull over the period of 4 years from birth to necropsy. The bars illustrate each sampling result and date for faecal (bottom), blood (central) and semen samples (top).

### Biopsy

At the age of 27 weeks (CW 31 in 2009), biopsy samples of the ileocaecal lymph nodes, caecum lymph nodes and distal jejunal lymph nodes were drawn at the Veterinary School of Hannover by routine endoscopic surgery.

### ***Dissection***

At the age of 4 years and 2 months (CW 15 in 2013), the bull was sedated with 10 ml Xylacin™ (20 mg/ml) and intravenously euthanized with 30 ml T61® (Intervet GmbH, Unterschleissheim, Germany). Forty-two tissue samples were collected during necropsy of the bull by professional veterinary staff (Table 3). They included tissue from the lymphatic system (No. 1–15), digestive tract and appending organs (No. 16–29), urogenital tract (No. 30–38), nervous system (No. 39 and 40) and thorax organs (No. 41 and 42). To avoid cross-contaminations, new, sterile surgical gloves, plastic forceps and scalpels were used for each tissue sample.

### ***Histopathology and immunohistochemistry***

Aliquots of the above-mentioned tissues were fixed with 4% neutral buffered formaldehyde and embedded in paraffin. Paraffin sections stained with haematoxylin and eosin were examined for histological lesions. Mycobacteria were labelled in paraffin sections by indirect immunoperoxidase staining using a polyclonal anti-MAP immune serum (Dako, Glostrup, Denmark) as described (Krüger et al., 2015). The primary antibody was replaced by an unrelated polyclonal antiserum in consecutive sections as negative control. Tissues from a cow with lesions of paratuberculosis were included as positive control.

### ***Culture***

*Mycobacterium avium* subsp. *paratuberculosis* growth in culture by standard procedures is a usual application in our routine diagnostic unit. The methods are accredited according to the standard DIN EN ISO/IEC 17025:2005. The preparation of faecal and tissue samples was performed as described in the official manual of diagnostic procedures published by the Friedrich-Loeffler-Institut (FLI), the German Federal Research Institute of Animal Health (Friedrich-Loeffler-Institut, 2014). However, the homogenization method used for necropsy tissue samples was modified, using the Precellys\_24 homogenizer. The preparation of the blood and semen samples was adapted on base of the official FLI protocol. The validity of the used method was confirmed for faecal samples by several inter-laboratory proficiency tests organized by the German FLI reference laboratory for mycobacteria. From the age of 7 weeks to necropsy, all collected faecal samples (n = 137) were prepared for cultivation, with the exception of 2 days (CW 33 and 49 in 2009). From CW 24 in 2010 to CW 41 in 2012,

semen samples (n = 87) were prepared for cultivation. Blood samples (n = 109) were included since CW 10 in 2011, too.

Faecal, semen, blood, biopsy and necropsy tissue samples were immediately prepared for bacterial cultivation on Herrold's egg yolkmedium with mycobactin (HEYM, BD BBL™, Heidelberg, Germany). The homogenizations of the biopsy tissue samples (1 g), taken from the 27-week-old calf, were carried out by trituration with sea sand. As opposed to this homogenization, the necropsy tissue samples (2.5 g) were transferred in 7-ml Precellys\_ tubes (Precellys\_ Ceramic Kit 2.8 mm, Peqlab, Erlangen, Germany), before 2.5 ml PBS was added and the samples were homogenized twice in a Precellys®24 homogenizer (Peqlab, Erlangen, Germany) for 30 s and 6000 rpm. Faecal (3 g), semen and blood (3–5 ml) samples were homogenized by thorough vortexing. For decontamination purposes, the prepared faecal aliquots and biopsy tissue homogenates were suspended in 30 ml of 0.75% hexadecylpyridinium chloride (HPC) solution, whereas the prepared semen and blood fluids as well as necropsy tissue homogenates were diluted with HPC solution using a ratio of 1:5. All preparations were mixed for 30 min and incubated for 24 h at room temperature. Afterwards, the supernatants were decanted down to 1 ml, before 0.2 ml of the sediments was inoculated to HEYM under sterile conditions and incubated at 37°C. From the faecal sediment three HEYM tubes and from blood, semen and tissue sediment two HEYM tubes were inoculated in parallel. In addition, faeces mixed with a MAP reference strain (ATCC 19698, Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as well as MAP-positive faeces from a clinically diseased cow were analysed as positive control samples. Bacterial growth was monitored for the first time after 4 weeks and afterwards regularly every 4 weeks for up to 16 weeks. Cultures exhibiting suspicious colonies were subcultured on HEYM – with and without mycobactin – to confirm slow growth rate and mycobactin dependence. Furthermore, growths of bacterial cultures were verified by a MAP-specific snPCR (Münster et al., 2013).

### ***DNA extraction***

DNA extraction and purification of all collected faecal, blood and semen samples for PCR analysis were performed using a modified protocol of the QIAamp Blood Kit (Qiagen, Hilden, Germany), as described by Münster et al. (2013). Faecal samples were

mixed 1:1 with PBS and filled into four aliquots of 1 g, respectively. Semen and blood samples without PBS were separated into two aliquots of 1 ml.

From tissue samples taken during biopsy and necropsy, DNA was extracted using a modified protocol of the QIAamp DNA Mini Kit (Qiagen). Purification of DNA from biopsy tissue samples was performed as described by Münster et al. (2011). The entire range of biopsy tissue materials was analysed (3 x 1 g of ileocaecal lymph nodes, 2 x 1 g of caecum lymph nodes, 6 x 1 g of distal jejunal lymph nodes). As low concentrations of MAP and the formation of clusters were expected, necropsy tissue samples were divided into 12 aliquots of 0.05 g, respectively. Only two aliquots were purified from the retropharyngeal lymph nodes and only ten aliquots from the pancreaticoduodenal lymph nodes, because little tissue material was available. All tissues were transferred to 2-ml Precellys\_tubes (Precellys<sup>®</sup> Ceramic Kit 1.4, 2.8 mm, Peqlab, Erlangen, Germany) containing 160 µl PBS. Soft tissue aliquots were homogenized for 30 s at 6500 rpm. Hard or elastic tissues were homogenized three times for 30 s at 6500 rpm in the Precellys<sup>®</sup>24 device, after which 200 µl tissue lysis buffer (ATL buffer) and 40 µl ready-to-use proteinase K solution (20 mg/ml) were added. Mixed samples were incubated on a thermomixer at 56°C and 900 rpm for 1 h followed by adding 400 µl lysis buffer (AL buffer). The tubes were incubated on the thermomixer again, but at 70°C and 900 rpm for 10 min. After centrifugation (20 000 g, 1 min), the supernatants were transferred into new tubes before 400 µl of ethanol (≥99.8%) was added. Loading of the QIAamp Spin Column and washing procedures were performed according to the manufacturer's instructions. Finally, the DNA was eluted in 200 µl elution buffer at 85°C and stored at -20°C prior to use.

### ***Qualitative semi-nested polymerase chain reaction***

A qualitative semi-nested PCR (snPCR), based on the insertion sequence IS900, was performed as described previously (Münster et al., 2011). The primers MAPfor1 (5'-GTC GGC GTG GTC GTC TGC TGG GTT GAT-'3) and MAPrev (5'-GCG CGG CAC GGC TCT TGT TGT AGT C-'3) amplified a 587-bp fragment in the first run. The forward primer MAP for 2 (5'- CGG GCG CAC GGT CCA TCA CG'3), together with the primer MAPrev, was taken for amplification of a 278-bp fragment in the second step. MAP-DNA extracted from the MAP reference strain ATCC 19698 was included as a positive control in each PCR run, whereas sterile distilled water was used as a



negative control. The snPCR used is well validated, and the primers were designed to bind to highly MAP-specific sites of the IS900 (Münster et al., 2011, 2013). The snPCR has a detection limit of 2.5 fg MAP-DNA (one genome equivalent/reaction). After PCR, 5 µl of the amplicons was mixed with 2 µl sample buffer and separated on 1% agarose gels for 1 h at 110 V. Subsequently, amplicons were stained with ethidium bromide (0.5 µg/ml) before PCR products were visualized under UV light. To preclude cross-contamination, DNA isolation, preparation of master mixes, addition of template DNA and electrophoresis were carried out in separate laboratories.

### ***Quantitative real-time polymerase chain reaction***

For quantification of MAP-DNA, a new quantitative realtime PCR (qPCR) assay based on the IS900 was developed (Czerny and Münster, 2013). The primers MAP<sub>523-542f</sub> (5'-TAC CGC GGC GAA GGC AAG AC-3') and MAP<sub>661-642r</sub> (5'-CGG AAC GTC GGC TGG TCA GG-3') were applied (MWG Biotech, Ebersberg, Germany) to amplify a 139-bp fragment. The TaqMan probe MAP<sub>617-636p</sub> (5'-FAM-ATG ACA TCG CAG TCG AGC TG-BHQ1-3') was designed to anneal on the PCR target allowing detection of specific PCR products. Amplification and fluorescence detection were performed on the Light Cycler<sup>®</sup> 480 System using 96-well PCR plates (Roche Molecular Diagnostic, Mannheim, Germany). A total reaction volume of 20 µl per well was used containing 10 µl Light Cycler<sup>TM</sup> 480 Probes Master (Roche Diagnostics, Mannheim, Germany), 0.5 µl of each upstream and downstream primer (10 pmol/µl), 1 µl MAP<sub>617-636p</sub>-probe (10 pmol/µl), as well as 3 µl sterile water and 5 µl of the extracted sample DNA. The amplification process started with an initial pre-incubation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 35 s. Fluorescence was measured within the FAM channel after the annealing step of each cycle. The qPCR was performed with all aliquots that tested MAP positive by snPCR. Samples were tested in three independent qPCR runs. Validation data have shown that the qPCR, under the used conditions, had a detection limit of 50 fg/reaction (10 genome equivalents/reaction) and a quantification limit of 5 pg/reaction MAP-DNA (945 genome equivalents/reaction). As a positive control and for the quantification of MAP-DNA concentration, an MAPDNA standard curve (MAP reference strain ATCC 19698) was titrated in log<sub>10</sub> steps (1 ng/µl to 1 fg/µl), whereas sterile distilled water was used as a negative control. MAP genome equivalents (MAP-GE) detected by qPCR were calculated from the MAP K-10 genome size of 4.829.781 bp (Li et al., 2005). Assuming

the molecular weight of one base pair is  $660 \text{ g} \times \text{mol}^{-1}$  and the Avogadro number is  $6.022 \times 10^{23} \text{ mol}^{-1}$ , calculations resulted in one MAP-GE of 5.29 fg of MAP-DNA. The formula for calculating the number of MAP-GE in blood or semen per ml and in faecal or tissue sample per g was as follows: Concentration of DNA (fg/ $\mu\text{l}$ )  $\times$  volume of eluted DNA (200  $\mu\text{l}$ )/ 5.29 fg  $\times$  1 (blood and semen sample);  $\times$  2 (faecal sample);  $\times$  20 (tissue sample) = MAP-GE per extracted sample aliquot, per ml or g sample, respectively.

### ***Enzyme-linked immunosorbent assay***

In total, 139 blood samples were analysed by a commercial indirect ELISA following the instructions of the manufacturer (IDEXX Paratuberculosis Screening, IDEXX Laboratories, Germany/ELISA Paratuberculosis Screening, Institute Pourquier, France). On 1 day of the investigation period, no ELISA could be performed (CW 44 in 2010). The evaluation of the serum samples was performed according to the cut-off recommended by the manufacturer: serum samples  $\leq 60\%$  S/P negative (-),  $>60\%$  –  $<70\%$  S/P borderline ( $\pm$ ),  $\geq 70\%$  S/P positive (+). Since 10 March 2010, the cut-off of the ELISA has been modified by the manufacturer: serum samples  $\leq 45\%$  S/P negative (-),  $>45\%$  –  $<55\%$  S/P borderline ( $\pm$ ),  $\geq 55\%$  S/P positive (+). Plates were read using an automated ELISA plate reader (Sunrise<sup>TM</sup>, Tecan Trading AG, Switzerland). Samples were analysed in duplicates, and the calculated S/P means were recorded.

### ***Statistical analysis***

Statistical analysis of the longitudinal data set was carried out by application of a linear logistic regression with a binary response variable, which is modelled as a binomial random variable ( $y_i$ ). The data were then analysed with the GLIMMIX procedure of SAS (Littell et al., 1999) using the following model:

$$\log \text{it} \left( \frac{\pi_r}{1 - \pi_r} \right) = \eta_r = \beta_0 + \beta_1 x_i$$

where  $\pi_i$  is the probability of detecting MAP-DNA in the respective matrix (faeces, blood or semen) from birth to necropsy by snPCR,  $\beta_0$  and  $\beta_1$  represent the logistic regression parameters, and  $x_i$  signifies the explanatory time variable. The regression lines are fitted by calculating back-transformed logistic regression parameters. The estimates based on the inverse link function  $\pi = \exp(x\beta) / [1 + \exp(x\beta)]$  to the original scale (probability).

In addition, the chi-square statistics ( $\chi^2$ ) and the corresponding Phi-coefficients ( $\phi$ ) were calculated to analyse the associations between the snPCR results of the MAP-DNA occurrence in faeces, blood and semen by snPCR using the FREQ procedure of SAS 9.4 software (SAS Inst., Inc., Cary, NC, USA, 2014). Calculation of the chi-square values was only performed if the frequencies were  $>5$ . Values of  $P \leq 0.05$  were considered significant for all tests. The Phi-coefficient is used to measure the strength of association between two binary variables. The level of association of the Phi-coefficient values was defined as follows:  $\phi = 0.00$  no association,  $0.00 > \phi \leq 0.25$  weak association,  $0.25 > \phi \leq 0.66$  moderate association,  $0.66 > \phi < 1.00$  strong association and  $1.00 =$  perfect association.

## Results

### *Longitudinal study*

The general condition of the investigated German Fleckvieh bull was good, and the faecal consistency was normal throughout the whole investigation period. The bull did not show any signs of paratuberculosis. However, it must be stated that the bull remained small in size for its age and showed poor growth performance similar to its father. On the day of the necropsy, with an age of 4 years and 2 months, the bull weighed 642 kg at a height of 140 cm.

### *Detection of MAP-DNA in faecal, blood, semen and biopsy tissue samples by snPCR*

At the first sampling day, at an age of 7 weeks (CW 11 in 2009), MAP-DNA was detected in faeces (Fig. 1). At the age of 27 weeks (CW 31 in 2009), biopsy samples were taken. No MAP-DNA was detected in any of the samples, including those from the ileocaecal lymph nodes, caecum lymph nodes and distal jejunal lymph nodes. At the age of 40 weeks (CW 44 in 2009), MAP-DNA was detected in blood for the first time. At the age of 2 years and 3 months (CW 17 in 2011), the pathogen was ascertained in semen for the first time. Over the course of the study, the results showed an intermittent MAP-DNA detection. Results are shown in detail in Fig. 1. In total, 23 of 140 (16.4%) blood samples and four of 89 (4.5%) semen samples tested positive for MAP-DNA by snPCR. The highest number of MAP-DNA-positive samples was detected in faeces (34/139; 24.5%) (Table 1). MAP-DNA was simultaneously detected in faeces and blood

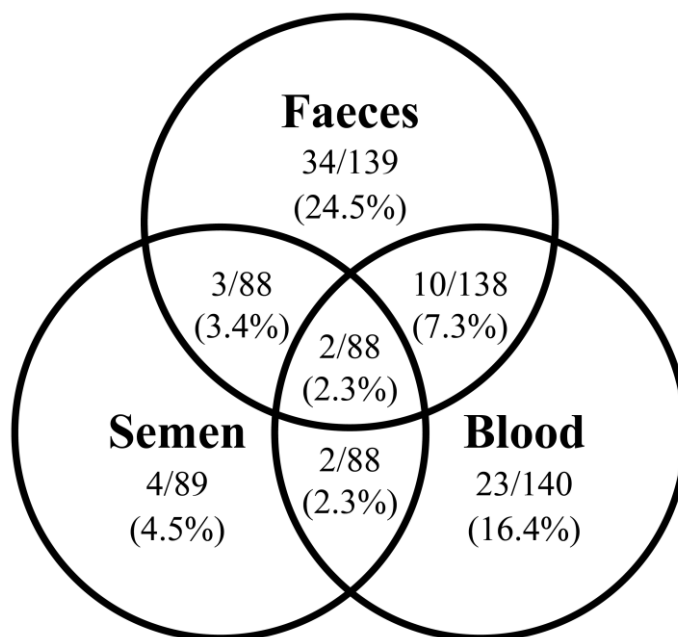
on 10 of 138 (7.3%) sampling days; in blood and semen on two of 88 (2.3%) days; and in faeces and semen on three of 88 days (3.4%). In semen, blood and faeces, MAP-DNA was detected simultaneously on two of 88 (2.3%) days (Fig. 2). MAP-DNA was not observed in any of the three matrices on 94 of 142 days (66.2%) and during intervals of up to 9 weeks (CW 12 to 20 in 2012) by snPCR.

**Table 1:** Frequencies of MAP-DNA detection by snPCR and the corresponding statistical analysis of the Chi-square test ( $\chi^2$ ) and Phi-coefficient ( $\phi$ ).

			Negative	Positive	Total (%)	$\chi^2$ p value	$\phi$
			<b>Faeces</b>				
<b>Blood</b>	Frequency (%)	Negative	93 (67.39)	23 (16.67)	116 (84.06)	6.67 P<0.01	0.22
	Frequency (%)	Positive	12 (8.70)	10 (7.25)	22 (15.95)		
	Total (%)		105 (76.01)	33 (23.91)	138 (100.00)		
			<b>Semen</b>				
<b>Blood</b>	Frequency (%)	Negative	77 (87.50)	2 (2.27)	79 (89.77)	n.a.	
	Frequency (%)	Positive	7 (7.95)	2 (2.27)	9 (10.23)		
	Total (%)		84 (95.45)	4 (4.55)	88 (100.00)		
			<b>Faeces</b>				
<b>Semen</b>	Frequency (%)	Negative	68 (77.27)	16 (18.18)	84 (95.45)	n.a.	
	Frequency (%)	Positive	1 (1.14)	3 (3.41)	4 (4.55)		
	Total (%)		69 (78.41)	19 (21.59)	88 (100.00)		

n.a. not analyzed

The matrices are indicated in bold.



**Fig. 2.** Venn diagram showing the interrelationships between the detectability of MAP-DNA in faeces, semen and blood of a naturally infected German Fleckvieh bull by snPCR. Given are the numbers of positive samples out of the total numbers of samples tested. The percentages of positive tested samples are given in parentheses.

#### ***Detection and quantification of MAP-DNA in faecal, blood and semen samples by qPCR***

The calculated average values of MAP-GE/g or ml, which are based on all positive aliquots and three independent qPCR runs of each sample, are listed in Table 2 in detail. In total, in five of 34 snPCR-positive faecal samples, MAP-DNA was detected by qPCR. In four of them, MAPDNA could be quantified. The amount of MAP-DNA ranged from  $2.61 \times 10^5$  to  $6.25 \times 10^6$  MAP-GE/g faeces. Detection but no quantification of MAP-GE was possible in one of the five qPCR-positive faecal samples due to the quantification limit of 5 pg MAP-DNA. In total, in 14 of 23 snPCR-positive blood samples, MAP-DNA was detected using qPCR. In seven of them, MAP-DNA was quantified ranging from  $4.07 \times 10^4$  to  $1.84 \times 10^5$  MAP-GE/ml. Detection but no quantification of MAP-GE was possible in the remaining seven blood samples. Furthermore, two of four snPCR-positive semen samples were positive by qPCR but quantification failed due to the quantification limit.

***Serological investigations***

Over the whole period of the investigation, 139 sera were tested by the Pourquier/Idexx-ELISA. The S/P ranged from 0.49% to 42.48% and, therefore, no antibody response was measured (serum samples  $\leq 45\%$  S/P negative). Therefore, the bull was a serological negative MAP shedder.

***Bacteriological investigation of faecal, blood, semen and biopsy tissue samples***

Over the whole investigation period, only three of 137 faecal samples investigated culturally showed moderate growth of MAP colonies on HYEM with mycobactin (Fig. 1). Furthermore, only from two of 109 culturally investigated blood samples MAP bacteria were isolated. No growth of MAP was ever observed in any of the culturally investigated semen samples ( $n = 87$ ). Furthermore, MAP was not cultivable from any of the biopsy tissues.

**Table 2.** Detection of MAP-DNA by qPCR in faeces, blood and semen collected from a subclinically infected German Fleckvieh bull over a period of 4 years from the age of 7 weeks to necropsy. Detection of MAP-DNA is given as average crossing point with standard deviation and the corresponding average values of MAP genome equivalents in calendar weeks for each year and each analysed matrix

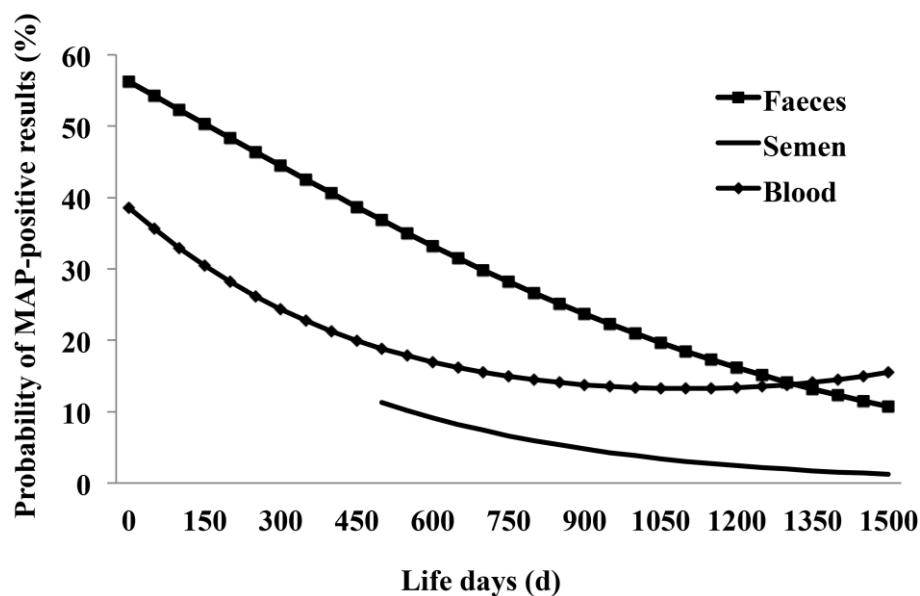
Matrix	Year	Calendar Week	Mean CP $\pm$ SD	MAP-GE/g or ml
Feces	2009	11	34.04 $\pm$ 1.67	positive*
		15	28.00 $\pm$ 0.40	1.52 x 10 <sup>6</sup>
		17	26.29 $\pm$ 0.40	6.25 x 10 <sup>6</sup>
		47	30.38 $\pm$ 0.19	2.61 x 10 <sup>5</sup>
	2012	50	28.66 $\pm$ 1.38	1.88 x 10 <sup>6</sup>
Blood	2009	44	31.16 $\pm$ 0.38	7.88 x 10 <sup>4</sup>
		45	30.59 $\pm$ 1.31	1.81 x 10 <sup>5</sup>
		46	32.37 $\pm$ 0.43	positive*
		47	31.99 $\pm$ 0.08	positive*
		49	32.64 $\pm$ 0.82	1.40 x 10 <sup>5</sup>
	2010	25	31.31 $\pm$ 0.40	6.49 x 10 <sup>4</sup>
		26	31.89 $\pm$ 0.06	4.07 x 10 <sup>4</sup>
	2011	17	31.64 $\pm$ 0.47	5.20 x 10 <sup>4</sup>
		20	35.00 $\pm$ 0.00	positive*
		27	35.00 $\pm$ 0.00	positive*
		33	35.00 $\pm$ 0.00	positive*
	2012	50	30.97 $\pm$ 1.11	1.84 x 10 <sup>5</sup>
	2013	6	35.00 $\pm$ 0.00	positive*
		11	35.00 $\pm$ 0.00	positive*
Semen	2011	17	32.06 $\pm$ 0.09	positive*
		20	32.03 $\pm$ 0.10	positive*

\* Detection but failure of quantification of MAP genome equivalents due to quantification limit of 5 pg MAP/DNA.

### Statistical analysis

To analyse the probability of detecting MAP-DNA by snPCR in the respective matrices (faeces, blood or semen) from birth to necropsy, a statistical analysis of the longitudinal data was carried out by applying a linear logistic regression with a binary response variable (Fig. 3). The probability of detecting MAP-DNA in faeces (56.24% to 10.73%) and semen (11.28% to 1.22%) decreased from birth to necropsy. The probability of detecting MAP-DNA in blood decreased from birth (38.53%) up to the age of 3 years (13.22%), and increased until the day of necropsy to 15.40%.

In addition, to analyse the statistical associations between the snPCR results of the MAP-DNA occurrence in faeces, blood and semen, the chi-square value ( $\chi^2$ ) was calculated in cases of frequencies  $>5$  (Table 1). For semen and blood as well as semen and faeces, no chi-square statistics could be performed, because the frequencies in both cases were  $<5$ . The calculated chi-square values between faeces and blood were greater than the critical value of 3.84 ( $P < 0.05$ ), indicating a statistical association. Following this, the corresponding Phi-coefficients ( $\phi$ ) were calculated to determine the measure of strength of the association between two matrices. A weak association was calculated between the matrices blood and faeces ( $\phi$  0.22;  $P > 0.05$ ;  $n = 138$ ).



**Fig. 3.** Statistical analysis of the MAP-DNA detection in the respective matrices (faeces, blood or semen) from birth to necropsy by snPCR was carried out by applying a linear logistic regression with a binary response variable.



***Dissection***

On 08 April 2013 (CW 15), the German Fleckvieh bull was dissected. During necropsy of the bull, no macroscopic abnormalities were observed, which were typical for an infection with MAP.

***Detection of MAP-DNA in tissue samples by snPCR***

In summary, MAP-DNA was detected by snPCR in 17 of 42 tissue samples. In Table 3, the results are listed in detail. In total, seven of 15 (46.7%) lymphoid organs tested MAP positive, including the right palatine tonsil, retropharyngeal lymph nodes, tracheobronchial lymph nodes, mediastinal lymph nodes, ileocaecal lymph nodes and superficial inguinal lymph nodes. The highest number of MAP-DNA-positive aliquots was detected in the bone marrow (9/12). Furthermore, MAP-DNA was detected in five of 14 (35.7%) tissues of the digestive tract, including tissues from the pancreas, duodenum, caecum, ileum and colon. None of the digestive tract appending organs, organ samples belonging to the head or any samples collected from the jejunum and rectum tested MAP-DNA positive. In total, five of nine (44.4%) organs of the urogenital tract were confirmed MAP positive by snPCR, including the testicle, epididymis, ampulla of deferent duct, prostate gland and kidney. No MAP-DNA was detected in the seminal gland, bulbourethral gland, adrenal gland or urinary bladder. Furthermore, the nervous system (cerebrum, cerebrospinal fluid) and thorax organs (heart and lung) also tested negative for MAP-DNA.

***Detection and quantification of MAP-DNA in tissue samples by qPCR***

Based on all positive aliquots and three independent qPCR runs of each tissue sample, the calculated crossing points – with standard deviations and average values of MAP-GE/g tissue – are listed in Table 3 in detail. In total, MAP-DNA was detected in seven of 17 snPCR positive tissue samples by qPCR, whereas MAP-DNA could only be quantified in two of them. The concentration of MAP indicated in the tracheobronchial lymph nodes was  $1.55 \times 10^6$  MAP-GE/g tissue and in the superficial inguinal lymph nodes  $3.86 \times 10^6$  MAP-GE/g tissue. MAP-DNA was detected, but due to the quantification limit it could not be quantified in the remaining five tissues, including the ileocaecal lymph nodes, pancreas, duodenum, colon and epididymis.

**Table 3:** Detection of MAP-DNA by snPCR and qPCR in tissue samples collected from a subclinically infected German Fleckvieh bull.

No.	Tissue samples	Results			
		snPCR	qPCR	Mean CP $\pm$ SD	MAP-GE/g
	n/N <sup>a</sup>	n/N			
<b>Lymphatic system</b>					
1	Spleen	0/12	-	-	-
2	Left palatine tonsil	0/12	-	-	-
3	Right palatine tonsil	1/12	0/1	negative	negative
4	Mandibular lymph nodes	0/12	-	-	-
5	Retropharyngeal lymph nodes	1/12	0/1	negative	negative
6	Tracheobronchial lymph nodes	2/2	1/2	32.02 $\pm$ 0.07	1.55 x 10 <sup>6</sup>
7	Mediastinal lymph nodes	1/12	0/2	negative	negative
8	Hepatic lymph nodes	0/12	-	-	-
9	Ileocecal lymph nodes	1/12	1/1	35.00 $\pm$ 0.00	positive*
10	Distal jejunal lymph nodes	0/12	-	-	-
11	Proximal jejunal lymph nodes	0/12	-	-	-
12	Colic lymph nodes	0/12	-	-	-
13	Pancreaticoduodenal lymph nodes	0/10	-	-	-
14	Superficial inguinal lymph nodes	2/12	2/2	31.39 $\pm$ 1.28	3.86 x 10 <sup>6</sup>
15	Bone marrow	9/12	0/9	negative	negative
<b>Digestive tract and appending organs</b>					
16	Tongue	0/12	-	-	-
17	Sublingual gland	0/12	-	-	-
18	Parotid gland	0/12	-	-	-
19	Liver	0/12	-	-	-
20	Bile	0/12	-	-	-
21	Pancreas	3/12	3/3	35.00 $\pm$ 0.00	positive*
22	Duodenum	6/12	5/6	35.00 $\pm$ 0.00	positive*
23	Cecum	1/12	0/1	negative	negative
24	Ileum	1/12	0/1	negative	negative
25	Proximal jejunum	0/12	-	-	-
26	Medial jejunum	0/12	-	-	-
27	Distal jejunum	0/12	-	-	-
28	Colon	1/12	1/1	34.81 $\pm$ 0.26	positive*
29	Rectum	0/12	-	-	-

- not determined if sample was negative by snPCR.

<sup>a</sup> Number of positive aliquots/number of tested aliquots of a tissue sample (n/N).

\* Detection but failure of quantification of MAP genome equivalents due to quantification limit of 5 pg MAP/DNA.

**Table 3:** continued

No.	Tissue samples	Results			
		snPCR	qPCR	Mean CP $\pm$ SD	MAP-GE/g
		n/N <sup>a</sup>	n/N		
Urogenital tract					
30	Testicle	4/12	0/4	negative	negative
31	Epididymis	3/12	1/3	35.00 $\pm$ 0.00	positive*
32	Ampulla of deferent duct	1/12	0/1	negative	negative
33	Seminal gland	0/12	-	-	-
35	Bulbourethral gland	0/12	-	-	-
36	Kidney	2/12	0/2	negative	negative
37	Adrenal gland	0/12	-	-	-
38	Urinary bladder	0/12	-	-	-
Nervous system					
39	Cerebrum	0/12	-	-	-
40	Cerebrospinal fluid	0/12	-	-	-
Thorax organs					
41	Heart	0/12	-	-	-
42	Lung	0/12	-	-	-

- not determined if sample was negative by snPCR.

<sup>a</sup> Number of positive aliquots/number of tested aliquots of a tissue sample (n/N).

\* Detection but failure of quantification of MAP genome equivalents due to quantification limit of 5 pg MAP/DNA.

### ***Histopathology and immunohistology in tissue samples***

Lesion characteristics of paratuberculosis were not detected in any of the examined tissue samples. Small groups of pigment-laden macrophages were present in the cortex of hepatic, tracheobronchial, pancreaticoduodenal, distal and proximal jejunal, colici and ileocaecal lymph nodes. Small round foci of mineralization and not demarcated by inflammatory cells were found in one jejunal lymph node and the left tonsil. Small multifocal abscesses with extensive central necrosis, feed plant material, bacterial colonies and surrounded by neutrophils were located deep in the crypts of the left tonsil. Specific labelling for mycobacteria was seen in close association to the plant material and the surrounding neutrophils of these abscesses. All other tissues were negative for mycobacterial antigens.

### ***Bacteriological investigation in tissue samples***

During the necropsy of the bull, 42 tissue samples in total were immediately prepared for bacterial culture, but MAP colonies could not be cultivated on HEYM with mycobactin after 16 weeks.

### **Discussion**

The infection of cattle with MAP starts mainly during the first months of life as a result of contact between newborn calves and contaminated colostrum, milk or faeces. Although excretion of MAP in the faeces and colostrum of its mother was not monitored during that period and an intrauterine infection cannot be ruled out completely, because no diagnostic data were collected from the calf immediately after birth, it is most likely that the bull investigated here over a period of 4 years was obviously infected naturally with MAP by its subclinically infected parents. The repeated detection of MAP-DNA in faeces samples of its father with concentrations of up to  $1.5 \times 10^5$  MAP-GE/ g faeces during the suckling period (Münster et al., 2013) supports an infection by the faecal–oral route or by bacterial loaded air. MAP-DNA was detected repeatedly in the examined bull with concentrations of up to  $6.25 \times 10^6$  MAP-GE/g faeces between the ages of 7 and 16 weeks. Sporadic faecal shedding of MAP has been reported previously in experimentally infected calves younger than 8 months (Collins and Zhao, 1994; McDonald et al., 1999). However, it is difficult to differentiate between a true infection (active MAP shedding) and a pass through (passive MAP shedding) of MAP (van Roermund et al., 2007). In the examined bull, it is possible that the pathogen was shed passively during the first weeks of life. This assumption was confirmed by the MAP-negative biopsy samples, which were collected at the age of 27 weeks. True infection of the bull was confirmed by the detection of MAP-DNA in blood at the age of 40 weeks. From this point onwards, MAPDNA was detected repeatedly in high concentrations of up to  $1.81 \times 10^5$  MAP-DNA equivalents/ml over a period of 5 weeks in blood. This indicated that the pathogen has passed the boundary from the intestine to other body regions.

Following the infection with MAP, animals normally enter a silent phase for at least 2 years in which no clinical symptoms are evident. During this stage, the pathogen is intermittently shed in faeces, but most of the time it is below the detection limit of

conventional methods (Whitlock and Buergelt, 1996). In the present study, MAP-DNA was detected intermittently by PCR in all three matrices (faeces, blood and semen). Within the first 2 years, 13 of 29 faecal (44.83%) and 10 of 30 blood samples (33.33%) tested positive for MAP-DNA. Additionally, the regression analysis of MAP-DNA detection had offered a higher probability in faeces and blood within the first 2 years of life. With increasing age, the probability of MAP-DNA detection decreased in all three matrices. However, the analysis is based on the distribution pattern of one animal and individual differences in the reactions to MAP exposure are likely. Furthermore, to examine the association between the snPCR results of the MAP-DNA occurrence in faeces, blood and semen, the chi-square value and the corresponding Phi-coefficient were calculated. These analyses could not be performed for semen and blood or semen and faeces because in both cases the ascertained frequencies were below the required frequencies. The statistical analysis between faeces and blood suggested weak associations. However, it should be mentioned that the associations were based on the degree of agreement of the positive as well as negative results and the calculated associations were mainly due to the degree of agreement of the negative results. For this reason, the results showed no connection between the circulation of MAP-DNA in blood and the simultaneous detection of MAP-DNA in faeces, as assumed. Furthermore, no statement can be made about the most appropriate matrix for MAP detection. In this study, MAP-DNA was detected most frequently (24.5%) and also at highest concentrations of up to  $6.25 \times 10^6$  MAP-GE/g in faeces. However, in the longitudinal study of the bull's father published recently (Münster et al., 2013), MAP-DNA was most frequently observed in blood samples (51.7%), but with lower MAP-GE/ml of up to  $1.7 \times 10^5$ . In the present study, MAP-DNA was not detected in any of the three matrices (faeces, blood and semen) on 94 of 142 sampling days (66.2%) and during intervals of up to 9 weeks (CW 12 to 20 in 2012). Furthermore, the commercial ELISA used did not show any antibody response throughout the whole investigation period of the bull. These results agree with a recent study, in which 30 of 33 cows were classified as MAP-free by three commercial serum ELISAs, although their MAP infection was previously confirmed by culture and PCR (Khol et al., 2012). Such negative ELISA results could be explained by the fact that ELISAs often used in the routine diagnostic of paratuberculosis are highly specific but lack in sensitivity for MAP-infected animals, especially in the subclinical stage of the disease.

It is estimated that only 10% to 15% of cattle infected naturally with MAP will develop clinical symptoms. Most of them remain in the subclinical stage of the disease and excrete the pathogen intermittently (Olsen et al., 2002). The bull examined in our study was consistently clinically unremarkable during the entire study. At the time of necropsy, neither macroscopic nor histological findings consistent with a MAP infection or host reactions to MAP were observed, although PCR assays had shown a wide distribution of MAP-DNA in the host, including the tissues of the lymphatic system, digestive tract and urogenital tract. At first sight, such a macroscopic and histological picture in PCR-positive samples is unusual for a MAP infection. It is expected that in MAP-infected tissues, especially in the gastrointestinal tract, inflammatory processes would be present. In our study, the right tonsil was the only tissue where mycobacteria were proved by staining. However, these bacteria were interpreted as environmental mycobacteria because of negative MAP-PCR results and missing histological lesions. Extensive distribution of MAP in cattle tissues with no macroscopic lesions or clinical signs was also determined previously in cows (Brady et al., 2008). Moreover, other authors concluded that the distribution of MAP soon after the infection might be much more widespread in the host than commonly believed (Whitlock and Buergelt, 1996; Mortier et al., 2013). The distribution of MAP in organs distant from the gastrointestinal tract is different for each animal (Ayele et al., 2004). Here, MAP was not observed predominantly in the tissues of the bulls' gastrointestinal tract. If such uncommon distribution of MAP in the host depends on the pathway of the infection, it is not described yet in the literature.

Although it is generally accepted that MAP could be transmitted vertically from cow to foetus (Whittington and Windsor, 2009), information about the role of bulls in the distribution of MAP is rare. It has been argued that transmission of MAP via semen is unlikely due to the low amount of MAP in the ejaculates of infected bulls (Philpott, 1993; Buergelt and Williams, 2004). In the present study, MAP-DNA could also be determined in only four of 89 semen samples by snPCR and in only two of them detection of MAP-DNA was possible by qPCR, whereas quantification of MAP-GE failed due to the quantification limit. Cultivation of MAP colonies from this matrix was completely unsuccessful. Therefore, it is likely that the concentration of MAP was low in the investigated semen samples. However, Münster et al. (2013) demonstrated that MAPDNA was detectable repeatedly over a period of 3 years in concentrations of up to

$5.7 \times 10^5$  MAP-GE/ml in the semen of the bull's father, an also naturally infected, asymptomatic German Fleckvieh bull. In the present study, MAP-DNA was determined in the male reproductive organs of the bull after necropsy, including the testicles, epididymis, ampulla of deferent duct, prostate gland and superficial inguinal lymph nodes, the associated lymph nodes of the reproductive organs. In other studies, a wide distribution of MAP in the reproductive organs of bulls was also determined. But in contrast, these bulls showed clinical symptoms, or if they were asymptomatic, typical macroscopic abnormalities were found during necropsy, indicating an advanced stage of the disease (Larsen and Kopecky, 1970; Larsen et al., 1981; Ayele et al., 2004; Glawischnig et al., 2004; Khol et al., 2010). The question of whether or not semen can elicit an infection of the cow or foetus has not yet been investigated. If evidence did arise, it could be necessary to expand the breeding hygiene management to include MAP because of the direct and indirect contact of semen from a single bull with a high number of cows. Furthermore, infected bulls may represent a source of infection for other breeding bulls as it was shown for semen production units from Pakistan that 20.0% (47/235) of the breeding bulls and 33.3% (6/18) of the teaser bulls were infected with MAP by ELISA (Abbas et al., 2011).

In animals with clinical signs of paratuberculosis, the specificity of faecal culture is thought to reach 98% and the sensitivity of faecal culture is considered to reach 70%. In contrast, cultivation of MAP from subclinically infected animals is difficult, because the sensitivity decreases to 23–49%, and depends heavily on MAP shedding in faeces (Nielsen and Toft, 2008). In the present study, MAP growth was only found in three of 139 culturally investigated faecal and in two of 109 culturally investigated blood samples, over a period of 4 years. No MAP growth was observed in any of the bull's tissue samples and as mentioned before in semen samples. The longitudinal study of its infected but asymptomatic father showed also a great discrepancy between MAP detection by culture and PCR (Münster et al., 2013). Although in some samples of the investigated bull, high MAP-GE concentrations of up to  $1.81 \times 10^5$ /ml,  $6.25 \times 10^6$ /g and  $3.86 \times 10^6$ /g in blood, faecal and tissue samples, respectively, were measured by qPCR, the results in general show a low MAP content in the investigated samples because only a low number of the aliquots per sample were tested positive by snPCR. Additionally, this indicates an inhomogeneous distribution of MAP by formation of clusters. Also in a study that monitored a Limousin cattle herd for a period of 53 months, in a significantly

high percentage of cattle, MAP was detectable by an IS900 qPCR but not in culture, respectively (Kralik et al., 2014); especially in the case of rather small numbers of MAP, the loss of viability of the bacteria during sample decontamination may reduce the detectability of MAP in culture (Bradner et al., 2013). In a previous study, the sensitivity of culture was estimated with approximately  $10^3$  MAP cells/g faeces (Kralik et al., 2011). Besides diagnostic measurement uncertainties, this discrepancy could be due to existence of dormant bacteria. Microorganisms in this state failed to grow by standard bacteriological methods, but remained alive (Oliver, 2010). MAP has the ability to enter into a viable but non-cultivable (VBNC) state in the environment (Whittington et al., 2004) and under experimental cultivation conditions (Lamont et al., 2012). Further, MAP-related bacteria such as *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* can enter the VBNC state, too. For *Mycobacterium tuberculosis*, it could be demonstrated that, depending on the growth-stimulating factor in the sputum of infected patients, between 80 and 99.99% of the bacteria were in the VBNC stage (Mukamolova et al., 2010). Also, a recent study indicated that VBNC *Mycobacterium tuberculosis* bacteria persist within the CD271<sup>+</sup>/CD45<sup>-</sup> mesenchymal stem cells of the bone marrow, which provide a niche for dormant infection (Das et al., 2013) and seem to play a key role in the establishment of latent tuberculosis (Raghuvanshi et al., 2010). In the investigated bull, it was striking that MAP-DNA was also detected in a high number of the analysed bone marrow aliquots (9/12) by snPCR. The missing cultivation of MAP from these samples may also suggest the VBNC state.

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### Conflict of Interest

The authors do not have a personal or financial relationship with other people or organizations that could inappropriately influence or bias this paper. All authors have approved the paper for release and are in agreement with its content.

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CHAPTER II

Paper published

*Journal of Medical Primatology*

**Detection of *Mycobacterium avium* subsp. *paratuberculosis* by IS900 based PCR assays in non-human primates kept in Germany**

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**Abstract**

**Background** Due to a sporadic occurrence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in non-human primates (NHP), the susceptibility of different NHP to MAP should be investigated.

**Methods** Fecal and tissue samples (ileum, ileocecal lymph nodes, bone marrow) of 20 animals (seven species) were analyzed by IS900-based PCRs and sequenced. Samples of MAP PCR positive NHP were further cultivated.

**Results** MAP DNA was detectable in two animals; the ileum of a cottontop tamarin and the bone marrow of a common marmoset. Cultivation of MAP failed. Sequence analysis revealed 100% homology to the MAP-K10 sequence. Pathohistological examinations offered no direct correlation to a MAP infection.

**Conclusions** MAP was detected for the first time in a common marmoset. But as both NHP suffered from other diseases an asymptomatic infection with MAP was assumed. The detection of MAP in the bone marrow might play a role in establishing latent paratuberculosis, as known from tuberculosis.

**Keywords:** bacteriological infection; bone marrow; *Callithrix jacchus*; *Saguinus oedipus*; PCR



## Introduction

Non-human primates are susceptible to acute or chronic enteral infections with different mycobacteria. One common form of enteritis, so called John's disease or paratuberculosis is induced by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In general, this disease occurs in ruminants, but pathological and histological changes typical for paratuberculosis have also been reported in several non-ruminant species, such as rabbits, donkeys and non-human primates [1-4]. Characteristic for the pathological picture of paratuberculosis is a thickened intestinal mucosa with corrugation and an enlargement of regional lymph nodes. Histopathologically, paratuberculosis is characterized by a chronic progressive granulomatous inflammation of the intestinal tract. The reasons for death, is the development of profuse diarrhea accompany with proteinemia, causing dehydration and cachexia [5]. Such typical clinical symptoms for paratuberculosis were observed previously in two non-human primate species [2-3]. MAP was detected in different tissues, particularly in the gastrointestinal tract of a captive group of stumptail macaques (*Macaca arctoides*) [2] and a captive female mandrill (*Mandrillus shinx*) [3]. Furthermore, the pathogen was detected in 10 out of 25 (40%) or 2 of 25 (8%) fecal samples of rhesus macaques (*Macaca mulatta*) by microscopic examination or direct IS900-based PCR, respectively [6]. These 25 fecal samples were collected from six free-living colonies with multiple clinical conditions (coughing and stool loose) from North India. The presence of MAP in non-human primates, which are kept in a German zoological garden, was previously shown [7]. The authors detected MAP DNA in each pooled fecal sample from 3 different non-human primate species; cottontop tamarins (*Saguinus oedipus*), gelada baboons (*Theropithecus gelada*), and black-and-white ruffed lemurs (*Varecia variegata*). Genome equivalents between  $2.2 \times 10^3$  and  $9.6 \times 10^6$  MAP/g feces were measured using MAP-specific PCRs. Ascertained MAP genome equivalent concentrations of black-and-white ruffed lemurs were highest in comparison to other examined animal species, such as goats, cavies, parrots or antelopes. Nevertheless, the detection of MAP in feces does not allow for a statement about a true infection with active shedding of MAP in feces or a pass through with passive MAP shedding in feces only [8, 9]. An infection with MAP is given when the pathogen exceeds the intestinal mucosa barrier and is distributed within the local tissue or is transported via the blood and lymphatic system to other areas of the body [10]. This study aims to demonstrate a

possible MAP infection in different non-human primate species by comparable investigations of feces and tissue samples using two MAP-specific PCRs and culture.

## **Material and methods**

### ***Humans care guidelines***

Care and housing conditions of the animals, kept at the German Primate Center (DPZ), complied with the regulations of the European Parliament and the Council Directive on the protection of animals used for scientific purposes (2010/63/EU) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2010). The animals in the zoological gardens are kept in accordance with the guidelines of the European Union for the accommodation and care of wild animals in zoological gardens (1999/22/EG). Furthermore, all primate facilities follow the applicable German Animal Protection Law. The investigated non-human primates from the DPZ were involved in different experimental studies. All examined animals suffered from severe diseases or were euthanized after study end. For the present study, only leftovers were used.

### ***Animals and sampling***

A total of 20 non-human primates belonging to seven different species were examined (Table 1). Four animals lived in different German zoological gardens; the other 16 non-human primates were kept at the DPZ. Altogether, the investigated species included one cottontop tamarin (*Saguinus oedipus*; No. 1), one emperor tamarin (*Saguinus imperator*; No. 2), eight common marmosets (*Callithrix jacchus*; No. 3 to 10), one barbary macaque (*Macaca sylvanus*; No. 11), one long-tailed macaque (*Macaca fascicularis*; No. 12), seven rhesus macaques (*Macaca mulatta*; No. 13 to 19) and one guereza (*Colobus guereza*; No. 20). During necropsy, tissue samples from the ileum, ileocecal lymph nodes, bone marrow, as well as fecal samples, were collected under sterile conditions and were transferred in sterile collection tubes by professional veterinary staff at the DPZ. All samples were analyzed directly or stored at -20°C prior to the respective assays. To avoid fecal contaminations, the ileum was cleaned thoroughly by flushing with sterile PBS before examination.

**Table 1:** Species, age, sex, weight, origin, pathohistological findings of the gut and the cause of disease or death of all investigated non-human primates.

No.	Species (Latin)	Age (years)	Sex	Weight (g)	Origin	Pathohistological findings: gut	Cause of disease/death
1	Cottontop tamarin ( <i>Saguinus oedipus</i> )	n. s.	♀	516	GZG <sup>a</sup>	Mild lymphoplasmacellular enteritis	Septicaemia related to purulent endometritis induced by
2	Emperor tamarin ( <i>Saguinus imperator</i> )	n. s.	♀	592	GZG	Without pathohistological findings	Acute necrotising hepatitis induced by <i>Listeriosis</i>
3	Common marmoset ( <i>Callithrix jacchus</i> )	3,3	♀	401	DPZ <sup>b</sup>	Without pathohistological findings	Without special findings, end of study
4	Common marmoset ( <i>Callithrix jacchus</i> )	4,1	♀	260	DPZ	Mild diffuse erosive duodenitis; related to <i>Candidiasis</i>	Wasting Marmoset Syndrome
5	Common marmoset ( <i>Callithrix jacchus</i> )	4,2	♀	599	DPZ	Without pathohistological findings	Without special findings, end of study
6	Common marmoset ( <i>Callithrix jacchus</i> )	4,3	♂	295	DPZ	Granulomatous enterocolitis	Severe enterocolitis with ruptur
7	Common marmoset ( <i>Callithrix jacchus</i> )	5,1	♀	520	DPZ	Without pathological findings	Severe chronic nephropathia
8	Common marmoset ( <i>Callithrix jacchus</i> )	6,1	♀	387	DPZ	Without pathohistological findings	Without special findings, end of study
9	Common marmoset ( <i>Callithrix jacchus</i> )	6,5	♂	397	DPZ	Severe chronic enteritis	Severe enterocolitis
10	Common marmoset ( <i>Callithrix jacchus</i> )	13,1	♀	429	DPZ	Moderate chronic enteritis	Moderate enterocolitis

<sup>a</sup> GZG German Zoological Garden<sup>b</sup> DPZ German Primate Center

n.s. not stated

**Table 1:** continued

No.	Species (Latin)	Age (years)	Sex	Weight (g)	Origin	Pathohistological findings: gut	Cause of disease/death
11	Barbary macaque ( <i>Macaca sylvanus</i> )	n. s.	♀	8861	GZG	Without pathohistological findings	Severe myocarditis and heart failure
12	Long-tailed macaque ( <i>Macaca fascicularis</i> )	5,1	♂	5873	DPZ	Moderate subacute colitis	Without special findings, end of study
13	Rhesus macaque ( <i>Macaca mulatta</i> )	n. s.	♀	2190	DPZ	Severe catarrhal colitis	Tularemia
14	Rhesus macaque ( <i>Macaca mulatta</i> )	2,2	♂	2292	DPZ	Severe catarrhal enteritis	Campylobacteriosis
17	Rhesus macaque ( <i>Macaca mulatta</i> )	3,3	♂	4691	DPZ	Without pathohistological findings	Severe polytrauma after innerspecies aggression and
15	Rhesus macaque ( <i>Macaca mulatta</i> )	4,5	♀	6361	DPZ	Without pathohistological findings	Severe polytrauma after innerspecies aggression and
16	Rhesus macaque ( <i>Macaca mulatta</i> )	5,3	♀	3483	DPZ	Severe ulcerative transmural enteritis induced by <i>E. coli</i>	Severe enterocolitis
18	Rhesus macaque ( <i>Macaca mulatta</i> )	7,2	♂	10251	DPZ	Without pathohistological findings	Without special findings, end of study
19	Rhesus macaque ( <i>Macaca mulatta</i> )	24,2	♂	14831	DPZ	Without pathohistological findings	Without special findings, end of study
20	Guereza ( <i>Colobus guereza</i> )	0,8	♂	2299	GZG	Without pathohistological findings	Severe purulent leptomeningitis, Listeriosis

<sup>a</sup> GZG German Zoological Garden<sup>b</sup> DPZ German Primate Center

n.s. not stated

***DNA extraction***

DNA extraction and purification of all collected fecal and tissue samples was performed using a modified protocol of the QIAamp Blood Kit and QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), respectively. Two aliquots of 50 mg of the sample matrices were transferred to 2 ml Precellys® tubes (Precellys® Ceramic Kit 1.4, 2.8 mm, Peqlab, Erlangen, Germany) containing 160 µl 5% (w/v) SDS solution. Fecal and tissue samples were homogenized for 15 sec at 6000 rpm and 30 sec at 6500 rpm in a Precellys® 24 homogenizer (Peqlab, Erlangen, Germany), respectively. Homogenized samples were incubated in a thermomixer at 37°C and at 900 rpm for 30 min. Further purification of the tissue samples was performed, as described elsewhere [11]. To the homogenized fecal samples 400 µl lysis buffer (AL buffer) and 40 µl ready-to-use proteinase K solution (20 mg/ml) were added. Mixed samples were incubated in a thermomixer at 56°C and at 900 rpm for 30 min, followed by a further 15 min at 95°C. After centrifugation ( $20000 \times g$ , 1 min), the supernatants were transferred into new tubes before 400 µl ethanol ( $\geq 99.8\%$ ) was added. Loading of the QIAamp Spin Column and washing procedures were performed according to the manufacturer's instructions. Finally, the DNA of the fecal and tissue samples was eluted in 100 µl elution buffer at 85°C, and stored at -20°C prior to use. To ensure contamination-free DNA preparation, a negative control was included during each DNA extraction by adding 50 µl sterile distilled water instead of the sample matrix.

***Qualitative and quantitative IS900 polymerase chain reactions***

Qualitative semi-nested PCR (snPCR) and quantitative real-time PCR (qPCR) were performed as described elsewhere [11, 12]. Amplification of DNA by snPCR resulted in fragment lengths of 587 bp and 278 bp, by qPCR in 139 bp, respectively. MAP DNA, extracted from the MAP reference strain ATCC 19698 (Leibnitz Institute DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), was included as a positive control in each PCR run, whereas sterile distilled water was used as a negative control. MAP genome equivalents (MAP-GE) detected by qPCR were calculated on the base of the MAP K-10 genome size (4.829.781 bp) [13]. One MAP-GE is calculated about 5.29 fg of MAP-DNA, assuming that the molecular weight of one base pair is  $660 \text{ g} \times \text{mol}^{-1}$  and the Avogadro number is  $6.022 \times 10^{23} \text{ mol}^{-1}$ . The number of MAP-GE in fecal or tissue sample per g were calculated by the following formula: Concentration of DNA (fg/µl)  $\times$  volume of eluted DNA (50 µl) / 5.29 fg  $\times$  20

(50 mg fecal or tissue sample) = MAP-GE per extracted sample aliquot, per ml or g sample, respectively. The detection limit of the snPCR was 2.5 fg MAP DNA/reaction (approximately 1 genome equivalents/reaction) [14, 15], whereas the qPCR had a detection limit of 50 fg/reaction (approximately 10 MAP genome equivalents/reaction) and a quantification limit of 5 pg MAP DNA/reaction (approximately 950 MAP genome equivalents/reaction) [11, 12]. Due to the lower sensitivity of the qPCR, only aliquots that tested positive for MAP by snPCR were further analyzed by qPCR in three independent qPCR runs.

### ***Cloning and sequencing of partial IS900 sequences***

The 587 bp fragments obtained from snPCR, covering nucleotide positions 183 to 770 within the IS900, were cloned into the StrataClone™ PCR Cloning Vector and transformed into *E. coli*, according to the manufacturer's instructions (Agilent Technologies, Waldbronn, Germany). Afterwards, plasmid DNA was extracted from two clones per amplicon using QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany). Sequencing of plasmid inserts (both strand directions of each clone) was conducted by the Göttingen Genomics Laboratory (Germany). Sequences were compared to all sequences from GenBank entries ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using BLAST search. To monitor similarities within all generated clone sequences, an alignment with published IS900 sequences was performed using the sequence analysis software “MegAlign” (DNA Star, Inc., Wisconsin, USA).

### ***Pathohistological investigations***

Samples for histopathology were collected during necropsies of animals sent for routine diagnostic to the pathology laboratory. Tissue samples were immersion fixed in 10% neutral buffered formalin. Tissues underwent routine histological processing. They were embedded in paraffin. Sections were stained with standard eosin-hematoxylin (HE) using the Varistain Gemini staining machine (Thermo Fisher, Scientific, Frankfurt/Main, Germany). Selected sections from the gastrointestinal tract were stained with Ziehl-Neelsen and Fite Faraco Kynjon using standardized protocols to demonstrate acid fast bacteria.

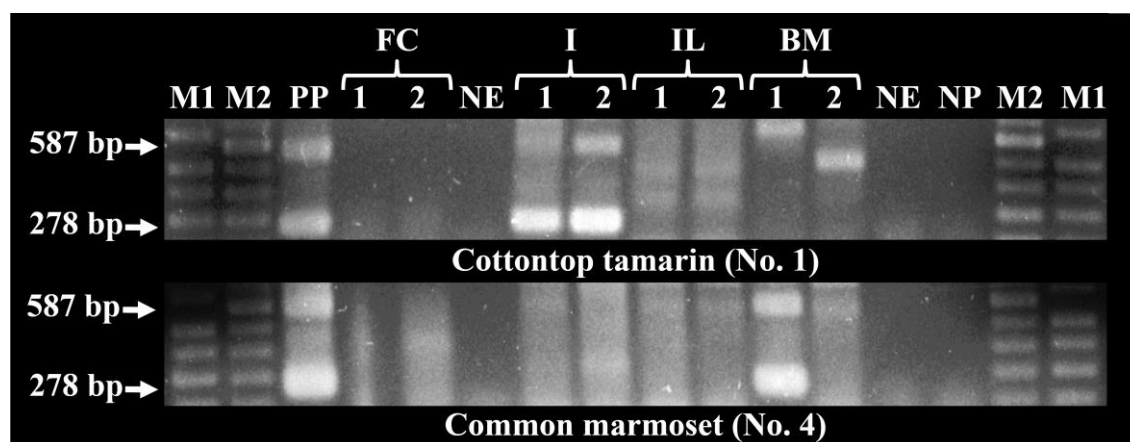
### ***Bacteriological investigations for MAP***

All four sample matrices (ileum, ileocecal lymph nodes, bone marrow and feces) from the non-human primates, which tested positive for MAP by PCR, were used for MAP cultivation. About 500 mg of feces and a maximum of 150 mg of tissue material could be prepared due to the small size of the new world monkeys. The procedure was carried out according to the official German manual of diagnostic procedures [16] using adapted amounts of reagents in relation to the sample volumes, and the tissue homogenization method was modified. Tissues were transferred to 2 ml Precellys<sup>®</sup> tubes (Precellys<sup>®</sup> Ceramic Kit 1.4, 2.8 mm, Peqlab, Erlangen, Germany) containing 150 µl PBS. All tissue aliquots were homogenized for 30 sec at 6000 rpm in a Precellys<sup>®</sup> 24 homogenizer. Fecal samples were homogenized by thorough vortexing. For decontamination purposes, the fecal and tissue homogenates were suspended in 5 ml of 0.75% hexadecylpyridinium chloride (HPC) solution. Further preparation steps were taken as described elsewhere [11, 16]. MAP cultures were performed in parallel on two tubecolumns of Herrold's egg yolk medium (HEYM, BD BBL<sup>™</sup>, Heidelberg, Germany) and in one modified Middlebrook 7H9 broth medium (M7H9C) [17]. In addition, negative as well as positive control fecal and tissue samples, mixed with the MAP reference strain ATCC 19698, were analyzed. Growths of bacterial cultures were monitored for the first time after four weeks and afterwards regularly every 4 weeks for up to 20 weeks. Cultures exhibiting suspicious colonies were verified by the above-mentioned snPCR.

## **Results**

### ***Detection and quantification of MAP DNA by PCRs***

In 2 out of the 20 (10%) investigated non-human primates, MAP DNA was detected by a MAP-specific IS900 based snPCR (Fig. 1). Specifically, MAP DNA was detected in the ileum of a female cottontop tamarin (*Saguinus oedipus*; No. 1) and in the bone marrow of a female common marmoset (*Callithrix jacchus*; No. 4). Both tissues also tested positive for MAP DNA by qPCR, but quantification failed, due to an amount below the quantification limit of 950 genome equivalents/reaction.



**Figure 1:** Detection of MAP DNA in a female cottontop tamarin (*Saguinus oedipus*; No. 1) and in a female common marmoset (*Callithrix jacchus*; No. 4) by snPCR, visualized by gel electrophoresis. Depending on the MAP DNA concentration, either the specific 278 bp fragment alone or the two 278 bp and 587 bp fragments were amplified and were responsible for a positive validation. M1, 1 kb molecular weight marker; M2, 100 bp molecular weight marker; PP, positive control of PCR (MAP-DNA); 1 and 2, each both PCR aliquots of sample matrix; FC, fecal sample; I, ileum sample; IL, ileocecal lymph nodes sample; BM, bone marrow sample; NE, negative control of the DNA extraction; NP, negative control of PCR (sterile distilled water).

### *Sequencing of partial IS900 sequences*

To confirm the specificity of the snPCR and in order to detect polymorphisms within the partial IS900 sequence, the 587bp DNA fragments from both samples, which tested positive by snPCR, were sequenced. A comparison between the analyzed sequences from the ileum of the cottontop tamarin (No.1) and the analyzed sequences from the bone marrow of the common marmoset (No.4) revealed a homology degree of 100% with the published bovine MAP-K10 IS900 reference sequence (GenBank: AE016958).

### *Pathological and histological investigations*

The main pathological and histological findings of the gut and the cause of disease or death for all investigated non-human primates are listed in Table 1. In general, no direct correlation between MAP and the individual cause of disease or death exists. Nevertheless, the pathological and histological findings of both non-human primates, which tested positive for MAP by PCR, were further described in detail. The cottontop



tamarin (No. 1) was found unconscious in a German zoological garden. The primate had a good nutritional condition. Main pathological and histological findings were observed in the reproductive system. The cause of disease and death was an acute purulent endometritis induced by a staphylococcus infection and followed by sepsis. Interestingly, mild, mainly plasmacellular duodenitis and ileitis were found in the small intestine. The mesenteric lymph nodes were also activated. Both were secondary findings unrelated to the disease. Acid fast bacteria were not demonstrable within the mucosa. Therefore, a direct correlation between MAP and the mild chronic enteritis remains questionable.

The common marmoset (No. 4) belonged to a group of primates from the DPZ and was euthanized due to its progressive weight loss. Pathologically striking were findings associated with the Marmoset Wastin Syndrome. Histologically, a mild erosive duodenitis was found, which was accompanied by the microbiological detection of *Candida*, as well as a mild cholecystitis.

### ***Bacteriological investigations for MAP***

In all culturally investigated matrices (ileum, ileocecal lymph nodes, bone marrow and feces) of both non-human primates, which tested positive for MAP by PCR, growth of MAP could not be observed after 20 weeks, neither on HEYM nor in M7H9C.

### **Discussion**

A few studies have shown so far that MAP could be observed in non-human primates [2, 3, 6, 7]. The first evidence for the presence of MAP in non-human primates kept in Germany was previously described in a screening study of pooled fecal samples from a zoological garden [7]. However, the detection of MAP in fecal samples could not confirm an infection, due to the fact that passive MAP shedding is possible. In this study, it was demonstrated that cottontop tamarins not only excrete MAP via feces [7], they are actually infected with MAP due to the detection of MAP DNA in the ileum. In rhesus macaques, neither an infection with MAP nor an excretion of MAP via feces could be shown in seven investigated animals, although MAP was already detected in feces of rhesus macaques previously [6]. Nevertheless, an infection of a common marmoset is demonstrated in this study for the first time due to the detection of MAP

DNA in the bone marrow. These findings indicate a prolonged spreading of MAP in different non-human primate species as known before [2, 3, 6, 7]. Whether non-human primates were responsible for the infection of other animals or not – for example primates in colonies or animals in zoological facilities – is not clear, but the list of animals, which are potential sources for MAP transmission, has been expanded.

Nevertheless, the possible distribution of MAP via feces of non-human primates was not measurable as part of this study, even though it is known from literature that MAP can be excreted in concentrations of  $10^6$  MAP genome equivalent/g feces from asymptomatic non-human primates [7]. Similar MAP concentrations were also measurable in feces of other animals asymptotically infected but it is known that the excretion of MAP appears usually intermittently [11, 14, 18]. Hence, it is admitted that diagnostic tests may lead temporarily to negative results during shedding-free intervals of the animals. This could potentially be an explanation for negative results in the analyzed fecal samples, especially in the case of the two MAP PCR positive non-human primates. However, diagnostic uncertainties may also occur due to low numbers of MAP cells (lower than the detection limit of the used method), limited sample volume or inhomogeneous distribution of MAP within the samples. In this study, the presence of MAP DNA was demonstrated in 2 out of 20 (10%) investigated non-human primates, but only in single matrices alone and by PCR analysis. Therefore, from the failure of quantification by qPCR it is obvious that the detectability of MAP was influenced by low numbers of MAP as well as low amounts of sample material.

Nevertheless, it is known that also other mycobacteria, like *Mycobacterium simiae*, could cause paratuberculosis similar symptoms in non-human primates [19]. To exclude an infection with mycobacteria other than MAP and to monitor the MAP types, which are involved in the current two cases, the detected MAP DNA was further characterized. PCR amplicons were sequenced to differentiate MAP strains by unique single nucleotide polymorphisms (SNPs) in specific regions of the IS900. It has been shown that SNPs at positions 169 (T/C) and 216 (G/A) are characteristic of MAP strains isolated from sheep in comparison to cattle types [20, 21]. The 587 bp PCR product of the used snPCR covered the 216 position. Adenine peaks at these loci in the sequence of the PCR amplicons from the ileum of the cottontop tamarin and the bone marrow of the common marmoset gave the indication that the detected MAP sequences may belong to a cattle strain. Nevertheless, occasionally an adenine peak was also found in the sheep

type of MAP. Literature suggests that all isolates belonging to the non-sheep type of MAP are highly conserved and thus cluster as a homogeneous group [22]. Moreover, also isolates from humans are thought to cluster with bovine strains [23].

In contrast to ruminants, studies reporting on monogastric animals with clinical symptoms and/or pathological and histological changes, associated with a MAP infection, are rare. Only two reports of non-human primates were found describing typical granulomatous enteritis induced by MAP [2, 3]. With careful interpretation of histological and pathological findings, the determined low-grade inflammatory processes in the gastrointestinal tract and the activation of the lymphatic system in the investigated cottontop tamarin (No. 1) – in which MAP DNA was detected in the ileum – could be associated with a MAP infection. However, these changes were secondary findings unrelated to disease or the death of the animal. The pathological and histological changes observed in the common marmoset (No. 4) indicate an underlying Wasting Marmoset Syndrome (WMS), which could not be classified in detail. In this study, it was interesting that MAP DNA was detected in two marmoset species (*Callitrichidae*), since these species often develop chronic colitis in captivity [24]. This colitis is mainly related to the symptom-complex of the WMS, but also occurs independently. The clinical picture of diseased marmosets shows clear parallels with the Crohn's disease in humans. Due to the clinical and pathological similarities, cottontop tamarins were already used as an animal model for scientific studies on ulcerative colitis and Crohn's disease [25]. However, the frequently discussed etiological relationship between MAP and Crohn's disease [26], and the possible association with WMS, still remains unclear.

Moreover, it was striking that MAP DNA was detected in the bone marrow of the common marmoset (No.4). Only two other studies have previously reported the detection of MAP in the bone marrow. MAP was detected in the bone marrow of two stump-tail macaques (*Macaca arctoides*) by microscopic examination [2] and in a German Fleckvieh bull (*Bos primigenius taurus*) by PCR [11]. In particular, these findings are of great interest, since recent studies have indicated that a MAP-related pathogen, *Mycobacterium tuberculosis*, persists within the CD271<sup>+</sup>/CD45<sup>-</sup> mesenchymal stem cells of the bone marrow in a viable but non-culturable (VBNC) state, and seems to play a key role in the establishment of latent tuberculosis [27, 28]. Hence, bone marrow perhaps also provides a niche for latent infections with MAP in

non-human primates. Furthermore, MAP cells in the VBNC state may explain in part the failure of MAP growth in this study, as MAP was only detectable by PCRs. Although it is known that MAP merge into a VBNC stage [29, 30], it is highly likely that cultivation failed due to small sample volumes as well as the low numbers of MAP cells, which were found typically in asymptotically infected animals. Furthermore, also the HPC decontamination of the samples leads to a reduction of viability of the bacteria, especially in the case of rather small numbers of MAP [31]. The sensitivity of MAP cultivation from the feces of subclinically infected animals is considered to be 23-49%, whereas cultivation of MAP from animals with clinical signs of paratuberculosis is thought to reach a sensitivity of 98% [32]. Also in other studies, a discrepancy between the results of PCR and cultivation were observed [9, 11, 14, 33]. In one of these previous studies, 875 of 1906 (45.91%) investigated fecal samples from dairy cattle were tested positively for the presences of MAP DNA by an IS900 based real-time PCR. In contrast, growths of MAP colonies were only detected in 169 of 1906 (8.87%) fecal samples, whereby all these samples were also tested positively by the real-time PCR [33].

The results of the present study confirmed MAP infections in two non-human primates kept in Germany due to the PCR positive results in the tissue samples. The incidence of MAP infections seems to be low and there is no direct correlation between the demonstration of the agent and the individual cause of disease or death. Therefore, an asymptomatic infection was assumed in the two positive cases. Nevertheless, by detecting MAP in the common marmoset, the number of non-ruminants infected with MAP was expanded. In particular, non-human primates are genetically and physiologically most similar to humans. Detailed analysis of MAP-infected non-human primates could promote further understanding of mechanisms in bowel diseases associated with a MAP infection or of MAP distribution in infected hosts. The re-detection of MAP in the bone marrow highlighted that mesenchymal stem cells of the bone marrow could provide a niche for a latent paratuberculosis infection. Therefore, it is recommended that further studies include the examination of MAP in bone marrow, in addition to gastrointestinal tissues and lymph nodes.

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CHAPTER III

Paper under review

*Journal of Zoo and Wild Medicine*

**Detection of *Mycobacterium avium* subsp. *paratuberculosis* in rock hyraxes  
(*Procavia capensis*) imported from South Africa**

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**Abstract**

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) causes chronic, progressive and consecutively fatal enteritis, especially in ruminants. MAP distribution among wildlife is not yet clear. In this study, three wild-born rock hyraxes (*Procavia capensis*) had been imported from South Africa to a German zoological garden. During the quarantine period, four young animals were born. The wild-born animals showed symptoms of mild diarrhea shortly after their arrival in the zoological garden, but all routine parasitological and bacteriological tests performed were negative. Therefore, the animals were additionally tested for MAP infection. MAP DNA was detected by semi-nested PCR (snPCR) in a pooled fecal sample of the seven animals. Subsequent PCR analysis of the individual feces samples confirmed the excretion of MAP in two rock hyraxes (one wild-born and one born in captivity). Sequence analysis of the corresponding 278 bp amplicons revealed 100% homology to the reference MAP-K10 IS900 sequence. No antibody response against MAP was detected in the individual serum samples. MAP-specific post-mortem lesions were not observed by gross pathology and histology, neither after death nor after euthanization of the animals. Nevertheless, MAP was detected by snPCR and culture in the gastrointestinal tract, urogenital tract, cardiovascular system and/or respiratory system of three other animals of the group (one wild-born and two born in captivity). This study is the first report confirming MAP occurrence in rock hyraxes. Therefore, it is recommended that especially veterinarians and zoo employees consider rock hyraxes as a possible source of MAP infection for domestic livestock in South Africa and the valuable animal stock of zoological facilities.

**Key words:** bacterial culture, *Mycobacterium avium* subsp. *paratuberculosis*, PCR, *Procavia capensis*, rock hyraxes, wildlife, zoological garden

## Introduction

The rock hyrax (*Procavia capensis*) – also known as dassie rat – is one of the four species of the order *Hyracoidea*, which belong to the clade of *Paenungulata* together with elephants (*Elephantidae*) and sea cows (*Sirenia*). Rock hyraxes represent a species with a weight of 2.5 to 4 kg, widely found in South Africa and the Middle East. Due to the extermination of predators such as black-backed jackal and lynx during the early 1960s, the number of rock hyraxes increased exponentially. In some areas of South Africa, they were officially listed as vermin.<sup>15, 17</sup> Currently, the population trend is stable,<sup>2</sup> although it is regularly reported that rock hyrax invasions exist in residential areas.<sup>38</sup> Moreover, rock hyraxes pose an agricultural problem as they compete with domestic livestock for available grazing space.<sup>15, 17</sup>

First reports on mycobacterial infections of rock hyraxes were published more than fifty years ago,<sup>35, 36</sup> when the so-called dassie bacillus was detected. Since then, the dassie bacillus has repeatedly been isolated from rock hyraxes in South Africa,<sup>30, 35</sup> as well as in zoo facilities in Australia and Canada.<sup>4, 19</sup> The dassie bacillus was formerly classified as an acid-fast bacterium resembling *Mycobacterium microti*, the causative agent of tuberculosis in rodents.<sup>32</sup> However, recent genomic analyzes have demonstrated that the dassie bacillus is more closely related to chimpanzee bacillus, *Mycobacterium mungi* and/or *Mycobacterium suricattae*.<sup>6</sup> *Mycobacterium africanum* was also reported to cause infection in rock hyraxes,<sup>12</sup> but it should be mention that the used method is not able to differentiate between *Mycobacterium africanum* and the dassie bacillus. Therefore, it is possible that identified bacteria might also belong to the dassie bacillus. However, the detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was never described in rock hyraxes.

MAP is the infectious agent of paratuberculosis, which mainly affects small and large domestic ruminants. In ruminants the disease is characterized by a degenerative, chronic granulomatous inflammation of the intestinal tract, which is often accompanied by diarrhea, weight loss, reduced reproductive performance and eventually death.<sup>13</sup> In addition to domestic ruminants, MAP has also been reported to infect a broad range of other domesticated, wild, and zoo animals including non-ruminants.<sup>1, 11, 23</sup>

This study provides a detailed analysis of the presence of MAP in a group of rock hyraxes, which were once captured in South Africa and kept in quarantine at a German zoological garden.

## Materials and methods

### *Animal samples*

Three wild-born rock hyraxes (*Procavia capensis*; animals 3, 4, 5) were imported from South Africa to the Zoo Duisburg (51°26'12''N, 6°48'14''E) in North Rhine-Westphalia, Germany (Table 1). During the course of the examination, four rock hyraxes (animals 1, 2, 6, 7) were born in captivity. The animals were not released from quarantine during the entire investigation period from 2007 until 2009. The wild-born hyraxes showed repeatedly mild symptoms of diarrhea that started five days after their arrival at the zoological garden. Repeated routine parasitological and bacteriological examinations of their feces excluded pathogens and parasites causing diarrhea, such as *Salmonella* spp., *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., *Clostridium* spp., or Cryptosporidia. Therefore, an infection with MAP was considered. To prove this suspicion, a pooled fecal sample of all seven rock hyraxes was tested for MAP DNA by semi-nested PCR with a positive result. Afterwards, individual fecal and blood samples of the animals were collected. During the period from May 2008 to July 2009, three rock hyraxes (1, 2, 7) died and the other four animals (3, 4, 5, 6) were euthanized in the zoological garden as a result of their presumed infection status. Animal 7 died as a result of an acute cardiovascular failure during anesthesia. Animal 1 had acute diarrhea and died suddenly without significant findings. The unexpected death of animal 2 could not be explained because no clinical symptoms were observed. Although the carcasses were put immediately on ice and delivered within 24 h for post-mortem examinations to the Institute of Veterinary Medicine in Göttingen, tissue samples were observed to be moderately decayed in animals 1, 4 and 5, whereas animals 2, 3, 6 and 7 showed a higher degree of decay. This may be due to the dense coat of the rock hyraxes inhibiting to give off body heat. During necropsy, gross pathological changes were documented and different tissue samples from the gastrointestinal tract and appending organs, the cardiovascular system, respiratory system and urogenital tract were collected under

**Table 1:** Results of the semi-nested PCR and cultural examination of investigated rock hyraxes (*Procavia capensis*).

Animal	1	2	3	4	5	6	7
Sex	♀	♀	♀	♂	♀	♂	♂
Place of birth	Zoo Duisburg	Zoo Duisburg	South Africa	South Africa	South Africa	Zoo Duisburg	Zoo Duisburg
Date of Birth	February 2008	January 2008	Unknown	Unknown	Unknown	February 2008	January 2008
Date of death or euthanization	May 2009	July 2008	July 2009	July 2009	July 2008	Sep 08	May 2008
<b>Results of</b>							
<b>Matrix</b>	<b>Culture</b>	<b>PCR</b>	<b>Culture</b>	<b>PCR</b>	<b>Culture</b>	<b>PCR</b>	<b>Culture</b>
	<b>PCR</b>	<b>Culture</b>	<b>PCR</b>	<b>Culture</b>	<b>PCR</b>	<b>Culture</b>	<b>PCR</b>
Feces	n.d. / -	- / -	n.d. / -	- / -	- / +	n.d. / -	n.d. / +
Duodenum	<sup>a</sup> / +	- / -	n.d. / n.d.	n.d. / n.d.	- / -	<sup>c</sup> / -	n.d. / n.d.
Ileum	<sup>a</sup> / -	n.d. / -	n.d. / -	+ / +	- / -	<sup>c</sup> / +	n.d. / -
Jejunum	<sup>a</sup> / +	- / -	n.d. / -	n.d. / -	- / -	<sup>c</sup> / -	n.d. / n.d.
Caecum	<sup>a</sup> / -	- / -	n.d. / -	+ / +	- / -	n.d. / -	n.d. / n.d.
Colon	<sup>a</sup> / +	- / -	n.d. / -	- / -	- / -	+ / -	n.d. / -
Rectum	n.d. / n.d.	n.d. / -	n.d. / n.d.	n.d. / n.d.	- / -	n.d. / +	n.d. / -
Lnn. ileocaecalis	<sup>a</sup> / +	n.d. / -	n.d. / n.d.	n.d. / -	- / -	n.d. / +	n.d. / -
Heart	<sup>b</sup> / -	n.d. / -	n.d. / n.d.	n.d. / n.d.	- / -	n.d. / -	n.d. / -
Lung	<sup>b</sup> / +	n.d. / -	n.d. / n.d.	n.d. / n.d.	- / -	n.d. / -	n.d. / -
Liver	- / -	- / -	n.d. / -	- / +	- / -	n.d. / -	n.d. / -
Spleen	<sup>b</sup> / -	n.d. / -	n.d. / -	- / +	n.d. / -	n.d. / -	n.d. / -
Kidney	<sup>b</sup> / -	n.d. / -	n.d. / -	- / +	n.d. / -	n.d. / -	n.d. / -
Testicles	n.a. / n.a.	n.a. / n.a.	n.a. / n.a.	- / +	n.a. / n.a.	n.d. / -	n.d. / -
Uterus	<sup>b</sup> / +	n.d. / -	n.d. / -	n.a. / n.a.	n.d. / n.d.	n.a. / n.a.	n.a. / n.a.
Ovary	<sup>b</sup> / +	n.d. / -	n.d. / -	n.a. / n.a.	n.d. / -	n.a. / n.a.	n.a. / n.a.

n.a. = not available

n.d. = not done

<sup>a</sup> Cultural examination of a pool of duodenum, ileum, jejunum, caecum, colon and Ln. Ileocaecalis of animal 1<sup>b</sup> Cultural examination of a pool of heart, lung, spleen, kidney, uterus and ovary of animal 1<sup>c</sup> Cultural examination of a pool of duodenum, ileum and jejunum of animal 6

sterile conditions (Table 1). Due to autolysis, the tissue analyses were not performed uniformly for all animals (Table 1). Furthermore, samples of the gastrointestinal tract were transferred into a 10% formaldehyde solution for histopathological investigations. To avoid cross-contamination, new sterile surgical gloves, plastic forceps and scalpels were used for each tissue sample.

### ***DNA extraction***

DNA was extracted and purified from fecal samples in duplicates using a modified protocol of the QIAamp Blood Kit (Qiagen, D-40724 Hilden, North Rhine-Westphalia, Germany). Briefly, 50 mg feces were transferred into sterile 2 ml tubes, mixed with 100  $\mu$ l PBS and ultra-sonicated thrice for 10 sec at 20 kHz (Bendelin Sonoplus HD 2200, BANDELIN electronic GmbH & Co. KG, D-12207 Berlin, Germany). Subsequently, 360  $\mu$ l lysis buffer (AL-Buffer) was added, and the samples were incubated on a thermomixer at 37°C for 30 min with continuous mixing at 900 rpm. Afterwards, 60  $\mu$ l proteinase K solution (20 mg/ml) and 600  $\mu$ l lysis buffer were added. The tubes were then incubated on the thermomixer at 56°C with shaking at 900 rpm for 30 min, followed by further incubation for 15 min at 95°C and 900 rpm.

After applying the lysis steps, the following DNA precipitation with ethanol ( $\geq 99.8\%$ ), loading of the QIAamp Spin Column and two-step washing was performed according to the corresponding kit manual. Finally, DNA was eluted in 200  $\mu$ l elution buffer (AE-buffer) at 85°C and stored at -20°C until further use.

To extract the DNA from the tissue samples (50 mg) in duplicate, the QIAamp DNA Mini Kit (Qiagen, D-40724, Hilden, North Rhine-Westphalia, Germany) was used according to kit protocol.

### ***Qualitative semi-nested polymerase chain reaction***

A qualitative semi-nested PCR (snPCR) based on the insertion sequence “IS900” was performed as described previously.<sup>23, 24</sup> The primers MAPfor1 (5'-GTC GGC GTG GTC GTC TGC TGG GTT GAT-3') and MAPPrev (5'-GCG CGG CAC GGC TCT TGT TGT AGT C-3') amplified a 587 bp fragment in the first run, while the MAPPrev primer – together with the forward primer MAPfor2 (5'- CGG GCG CAC GGT CCA TCA CG'3) – produced a 278 bp fragment in the second run. As a positive control, DNA was extracted from a MAP reference strain ordered from the German Collection of

Microorganisms and Cell Cultures (DSMZ, D-38124, Braunschweig, Lower Saxony, Germany; ID: 44133). Sterile distilled water was used as a negative control.

### ***Cloning and sequencing***

To confirm MAP specificity, the 278 bp fragments obtained from snPCR of the fecal samples – covering nucleotide position 492 to 770 within the IS900 (GenBank: AE016958) – were inserted into the pCR® 2.1-TOPO® plasmid vector (Fisher Scientific GmbH, D-58239 Schwerte, North Rhine-Westphalia, Germany). Afterwards, the plasmid vector was transformed into *E. coli* according to the manufacturer's instructions (Wizard® Plus SV Minipreps Purification System, Promega, D-68199, Mannheim, Baden-Wuerttemberg, Germany). Plasmid DNA was isolated from two clones per amplicon using QIAprep® Spin Miniprep Kit (Qiagen, D-40724 Hilden, North Rhine-Westphalia, Germany). The inserts were sequenced in both strand directions using the M13 primers (The Goettingen Genomics Laboratory, D-37077 Goettingen, Lower Saxony, Germany). The obtained sequences were compared to all sequences from GenBank entries ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) by BLAST search. To monitor the degree of similarity, an alignment with published IS900 sequences was performed using "MegAlign" (DNASar, Inc., Madison, Wisconsin 53705, USA).

### ***Cultural examination of MAP***

MAP growth from fecal and tissue samples were carried out on Herrold's egg yolk medium (HEYM) obtained from Becton, Dickinson and Company (D-69126 Heidelberg, Baden-Wuerttemberg, Germany). The procedure was performed as described in the German reference protocol.<sup>9, 14</sup> In addition, feces spiked with a MAP reference strain (DSMZ ID: 44133) were used as a positive control. Growth of bacterial cultures was monitored for the first time after four weeks, and then regularly every four weeks for up to 16 weeks. Cultures exhibiting suspicious colonies were verified by the snPCR as mentioned above.

### ***Enzyme-linked immunosorbent assay***

To analyze the antibody response to MAP, a slightly modified procedure from a protocol published previously using the HerdChek M.pt Ab (IDEXX GmbH, D-71636 Ludwigsburg, Baden-Wuerttemberg, Germany) was applied.<sup>34</sup> Twenty microliters of the rock hyrax serum samples were mixed with 180 µl absorbent solution and incubated at RT for 60 min. The pre-incubated samples were transferred to the MAP antigen-coated

96-well microplate and incubated at RT for 60 min. The plate was washed five times with the wash solution and incubated for 60 min at RT with 100 µl of the absorbent solution. All wells were washed five times. Next, 100 µl horseradish peroxidase (HRP) protein-G conjugate, reacting with antibodies of multiple animal species, was added to each well and incubated for further 30 min at RT. The wells were washed five times again and 100 µl of the substrate solution was added. Directly, the plate was incubated in a closed dark chamber for 10 min at RT, before 100 µl of the stop solution were added. The optical density (OD) was measured at 450 nm wavelength to calculate the S/P ratios. The cut-offs were applied as recommended by the manufacturer for ruminants: serum samples  $\leq 0.15$  S/P negative (-),  $>0.15 - <0.3$  S/P borderline ( $\pm$ ),  $\geq 0.3$  S/P positive (+). The positive and negative controls supplied by the HerdChek M.pt Ab ELISA kit were used as described by the manufacturer.

### ***Histopathology and immunohistochemistry***

For histopathological examination, tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. The prepared sections were stained with standard eosin-hematoxylin (HE) using the Varistain Gemini staining device (Thermo Fisher, Scientific, D-64293 Darmstadt, Hesse, Germany). Sections of small and large intestines were stained with Ziehl-Neelsen using standardized protocols to detect acid-fast bacteria. In addition, immunohistochemistry was performed as described elsewhere using a rabbit polyclonal antibody against *Mycobacterium bovis* (DAKO, D-22083 Hamburg, Germany).<sup>5</sup>

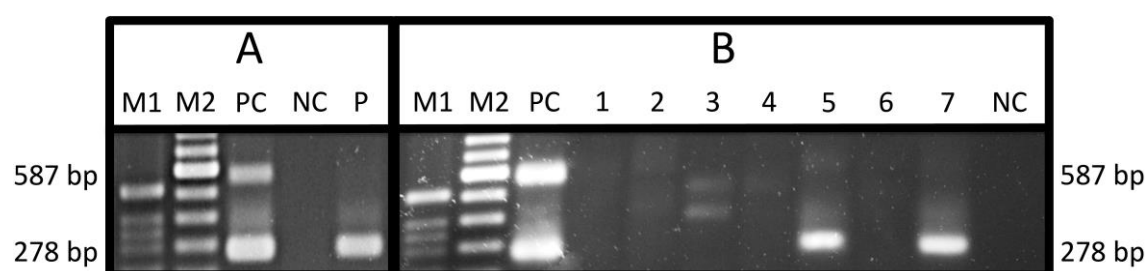
### **Results**

Physical condition, sensorium and eating habits of each animal were good and the fecal consistencies were normal most of the time, except for episodes of mild diarrhea. Due to the clinical symptomatic, the exclusion of several other diarrhea pathogens and the fact that rock hyraxes are susceptible to different mycobacteria, it was presumed that these group of rock hyraxes may be infected with MAP. In a pooled fecal sample from all colony members the presence of MAP DNA was confirmed by snPCR (Figure 1A). Afterwards, analysis of individual fecal, blood and tissue samples verified the occurrence of MAP in the rock hyraxes.



***Detection of MAP DNA in individual fecal and tissue samples by snPCR and sequencing of the PCR amplicons***

Analysis of individual samples revealed MAP DNA in the feces of animals 5 and 7, but not in their internal organs (Figure 1B, Table 1). Sequences of the DNA (278 bp) amplified from both samples were aligned to the MAP-K10 IS900 reference sequence (GenBank: AE016958). A homology degree of 100% was recorded. Interestingly, MAP DNA was detected in the tissue samples mainly in the gastrointestinal tract, but also in heart, lung, liver, spleen, kidney, and reproduction organs of three other rock hyraxes (1, 4 and 6) with negative results in the corresponding fecal samples (Table 1). Both fecal and tissue samples of the residual two animals (2 and 3), were tested negative for MAP in the snPCR.



**Figure 1:** Detection of MAP DNA in the pooled fecal sample (A) and individual fecal samples (B) collected from a group of seven rock hyraxes (*Procapra capensis*) by an IS900 based semi-nested PCR, visualized by gel electrophoresis. Depending on the MAP DNA concentration, either the two specific 587 bp and 278 bp fragments or the 278 bp fragment alone were amplified and demonstrate a positive finding. M1, 1 kb molecular weight marker; M2, 100 bp molecular weight marker; PC, positive control; lane P, the pooled fecal sample; lanes 1 to 7; individual fecal samples of rock hyrax 1 to 7; NC, negative control.

***Cultural examination of MAP in individual fecal and tissue samples***

Fecal and tissue samples were prepared for cultivation of MAP on HEYM. Unfortunately, due to the high degree of decay in the animals, no tissue samples from animals 3 and 7, as well as some single organ samples from the other animals, were prepared for the cultural examination (Table 1). MAP colonies were detected and

confirmed by snPCR in the tissue samples of two out of five investigated animals. Growth of MAP colonies was detected in the ileum and caecum tissue samples of animal 4, an imported animal. Furthermore, MAP was isolated from a pool of small intestine section samples and the colon of animal 6. MAP was not cultivable from samples of animals 1, 2 and 5.

### ***Serological investigations***

Serum samples of all rock hyraxes were analyzed by a commercially available ELISA (HerdChek M.pt Ab; IDEXX). Positive and negative controls confirmed the validity of the performed ELISA as specified by the manufacturer. The S/P ratio of the rock hyrax sera was below 0.15, and therefore, no antibody response was measured.

### ***Gross pathological, histopathological and immunohistochemical findings***

Five rock hyraxes (1, 3, 4, 5 and 7) were examined histopathologically. Although no typical, gross morphological changes, which may be related to a MAP infection, were observed, the histological examinations showed inflammatory cell infiltrates of the intestinal wall. In detail, the lamina propria and submucosa of the intestines of animal 5 contained numerous inflammatory cells including lymphocytes, neutrophilic and eosinophilic granulocytes. Some of the macrophages carried intracytoplasmatic eosinophilic structures, but MAP was not detected within these cells. Various submucosal locations showed secondary lymphoid tissue. Mesenteric lymph nodes were characterized by a moderate hyperplasia accompanied by accumulations of macrophages and sporadic giant cells. These results could be a sign of indicating minor subacute enteritis. Animal 1 had diarrhea and died suddenly. The intestinal mucosal presented extensive destruction, hindering adequate histologic analysis. Nevertheless, some intestinal sections showed moderate GALT hyperplasia and local presence of multiple small mineralized foci. Animals 3 and 4 showed similar alterations of the gut, the small and the large intestine were characterized by multifocal occurrence of lymphoplasmacytic inflammation and an increased presence of eosinophil granulocytes in the lamina propria. In the lamina propria and submucosa of the small intestine, multifocally lymphocytes, macrophages and polymorphonuclear neutrophils and eosinophils were observed. The small and large intestine of animal 7 were without any significant findings. Acid-fast bacteria could not be detected in the tissue of any animal by Ziehl-Neelsen staining. The results of the immunohistochemical examinations of the

intestinal tract were invalid, due to a large number of autolysis-induced nonspecific findings.

## Discussion

Studies on infections of rock hyraxes with mycobacteria other than MAP have already been published.<sup>4, 12, 19, 30, 35, 36</sup> Nevertheless, this is the first report on the isolation of MAP in these animal species. MAP was detected in the gastrointestinal tract, urogenital tract, cardiovascular system and/or respiratory system of three rock hyraxes (one wild-born and two born in captivity) and in the feces of two others (one wild-born and one born in captivity) by snPCR and/or culture. The 278 bp amplicons of the detected DNA in the fecal samples were identical to the IS900 reference sequence of the MAP-K10 Genome (GenBank: AE016958), confirming the identity of MAP.

Although in most rock hyraxes (5 animals) MAP was detected, both gross pathological and pathohistological findings did not show any distinctive signs of paratuberculosis. However, these investigations were impeded by the autolysis of the carcasses whereby the validity of the results might be negatively influenced. The only clinical finding, which may be related to an infection with MAP, was the mild, irregular diarrhea of the investigated animals. These findings are similar to the early phase or subclinical infections described in cattle.<sup>8, 25, 37</sup> Symptoms are mild and restricted to irregular, short periods of soft feces accompanied by the excretion of low amounts of MAP. While paratuberculosis is well documented for domestic cattle, little is known about pathogenesis and course of the infection in non-ruminants. In previous studies on non-ruminant species such as foxes (*Vulpes vulpes*), stoats (*Mustela erminea*), weasels (*Mustela nivalis*), crows (*Corvus corone*), and wood mice (*Apodemus sylvaticus*) infected with MAP were reported. However, in these animals mostly no or just mild gross pathological changes and histopathological lesions were found.<sup>1, 3, 11</sup> Therefore, it could be assumed that MAP may replicate in rock hyraxes without triggering characteristic macroscopic or microscopic lesions. Up to now, the only non-ruminant species, which is known to develop typical histopathological changes for paratuberculosis, is the rabbit (*Oryctolagus cuniculus*).<sup>3</sup>

It is assumed that paratuberculosis is present in most countries.<sup>26</sup> Especially in South Africa, the disease seems to be an emerging problem in sheep.<sup>20</sup> Therefore, it is most

likely that the wild-born rock hyraxes were naturally infected in South Africa and were responsible for the infection of the young animals, which were born in captivity. Improbable, but not completely excludable is an infection of the wild-born rock hyraxes with MAP whilst being transported from South Africa to Germany and/or during the quarantine period, although strict hygiene interventions were met. However, only very little information are available on the occurrence and host range of MAP in Africa. Sheep,<sup>20, 29</sup> goats,<sup>20, 22, 27</sup> camels,<sup>27</sup> as well as exotic and local breeds of cattle are known to be domesticated hosts of MAP in Africa.<sup>21, 28</sup> To our knowledge, the only record of paratuberculosis in the African wildlife is the serological detection of MAP antibodies in antelopes (20/373) from Zambia.<sup>16</sup>

In other regions of the world, it is known that besides several ruminant wildlife species, non-ruminant species are also reservoirs for MAP, such as wild rabbits in Scotland.<sup>1, 3, 11</sup> Especially in East Scotland, the high prevalence of MAP in rabbits is associated with difficulties in controlling paratuberculosis on cattle farms.<sup>31</sup> Therefore, rock hyraxes may play a role in the indirect transmission of MAP to domestic ruminants in South Africa since they share the same grasslands.<sup>15, 17</sup> For epidemiological tracing of species transmission between wild and domestic animals or different livestock herds, multitarget genotyping seems to be a suitable tool.<sup>10, 33</sup> By the combination of different tools like short sequence repeat (SSR) analysis, mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) analysis, and restriction fragment length polymorphism analysis based on IS900 (IS900-RFLP) a transmission of MAP between wild-living red deer (*Cervus elaphus*) and farmed cattle herds, which shared the same habitats, was demonstrated previously.<sup>10</sup> Therefore, this should further be investigated in rock hyraxes sharing grassland with domesticated ruminants in Africa.

In addition to the potential risk of transmission to the domestic livestock, previous research data shows that captured wild animals may be a source of the infection for the valuable animal stock of zoo facilities.<sup>23, 34</sup> In particular, this relates to the introduction of new zoo animals infected subclinically with MAP. The long incubation period without visible clinical signs result in a significant unrecognized spread of the bacteria to a former paratuberculosis-free collection. While MAP-infected domestic ruminants were usually culled,<sup>18</sup> this method is not suitable for zoo animals, especially for endangered species. Therefore, a recommended focus is on the prevention of the infection by enforcing strict hygiene standards during the quarantine period. In addition,

standard hygiene measures such as restricting access to the animal enclosure for a few authorized staff members, and using protective clothing and utensils must be applied.

An essential requirement for preventing and controlling paratuberculosis is the rapid identification of asymptomatic MAP shedders. This strategy is necessary for animal stocks of zoo facilities as well as for livestock farms. Likewise, the introduction of MAP infected animals from exogenous sources was prevented by the verification of the MAP infection status during the quarantine period. For such a survey, the analysis of pooled fecal samples seems to be an ideal approach, since this is an economical, as well as a rapid procedure for identifying MAP excretion. Of course, the implementation of individual sampling is not always possible in the daily zoo and farm routine due to time and economic reasons.

In one previous study, antibodies against MAP were detected successfully in eleven different zoo animal species by the same ELISA as used in the present study, including one non-ruminant species, the Malayan tapir (*Tapirus indicus*).<sup>34</sup> This indirect ELISA assay based on a protein G conjugate, which is known to react with antibodies of multiple animal species and seems to be a useful alternative to species-specific secondary antibodies, especially for the examination of exotic animal species. In contrast, in our investigations, no antibody response against MAP was measurable. Generally, the presence of an antibody response during the early phase of the MAP infection is infrequent.<sup>7</sup> Furthermore, it is known that ruminants could develop no antibody response at all or an inconsistent antibody response against MAP.<sup>8, 25</sup> Currently, neither antigenic nor serological detection methods are the most favourable option for the diagnosis of paratuberculosis during the subclinical stage of the disease. Therefore, combinations of both methods, as well as several repetitions at different time points at individual level, are necessary for a reliable diagnosis of paratuberculosis.

## Conclusion

To our knowledge, the present study is the first report on the detection of MAP in rock hyraxes. The results indicate that MAP is a pathogen of free-living rock hyraxes, which represents a potential source of infection for the valuable animal stock of zoo facilities and may play a role as a natural MAP-reservoir in South Africa. Therefore, it is our recommendation that especially veterinarians and zoo employees should consider the

possibility of a MAP infection in animals captured in the wild. It may also be advisable to evaluate the MAP infection status of rock hyraxes in order to control the spread of MAP.

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**CHAPTER IV**

**Paper published**

*Plos One*

**Development of a recombinase polymerase amplification assay for rapid detection  
of *Mycobacterium avium* subsp. *paratuberculosis***

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Short title: Rapid detection of *Mycobacterium avium* subsp. *paratuberculosis*

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**Abstract**

**Background:** The detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections in ruminants is crucial to control spread among animals and to humans. Cultivation of MAP is seen as the gold standard for detection, although it is very time consuming and labour intensive. In addition, several PCR assays have been developed to detect MAP in around 90 minutes, but these assays required highly sophisticated equipment as well as lengthy and complicated procedure.

**Methodology/Principal Findings:** In this study, we have developed a rapid assay for the detection of MAP based on the recombinase polymerase amplification (RPA) assay targeting a MAP specific region, the IS900 gene. The detection limit was 16 DNA molecules in 15 minutes as determined by the probit analysis on eight runs of the plasmid standard. Cross reactivity with other mycobacterial and environmentally associated bacterial strains was not observed. The clinical performance of the MAP RPA assay was tested using 48 MAP-positive and 20 MAP-negative blood, sperm, faecal and tissue samples. All results were compared with reads of a highly sensitive real-time PCR assay. The specificity of the MAP RPA assay was 100%, while the sensitivity was 89.5%.

**Conclusions/Significance:** The RPA assay is quicker and much easier to handle than real-time PCR. All RPA reagents were cold-chain independent. Moreover, combining RPA assay with a simple extraction protocol will maximize its use at point of need for rapid detection of MAP.

**Keywords:** *Mycobacterium avium* subsp. *paratuberculosis*; recombinase polymerase amplification assay; point of need

## Introduction

Paratuberculosis (Johne's disease) is caused by the Gram-positive, aerobic, non-motile, non-spore-forming and acid fast *Mycobacterium avium* subsp. *paratuberculosis* (MAP) [1]. The disease is characterized by a chronic progressive course marked by emaciation and accompanying lethal enteritis. MAP infects primarily large and small ruminants leading to diarrhea, weight loss and decreased milk production [2]. Particularly, animals infected subclinically represent a hotspot for transmitting MAP within the herd [3]. In humans, MAP was isolated from patients with Crohn's disease (inflammatory bowel disease) [4-6] and for more than 100 years, MAP has been discussed to be the causative agent [7]. Johne's disease is leading to huge economic losses in dairy production and since no pharmacological treatment or a licensed vaccine are available, early detection of the source of a MAP infection in a herd is very crucial to diminish such losses.

The gold standard for the diagnosis of MAP is culturing the bacteria [8], which takes up to 12 weeks and is only possible in highly equipped laboratories. Direct detection of MAP antibody with ELISA has been extensively applied but the clinical sensitivity and specificity is lower than the molecular assays [8]. Many real-time, conventional, semi-nested and nested PCR assays have been developed for the detection of MAP in up to 3 hours [9-11]. Nevertheless, a big challenge is the implementation of PCR in mobile point of need systems, because of its rapid thermocycling between the denaturation temperature, 95°C, and approximately 50°C for primer annealing together with a precise temperature control [12].

Unlike PCR, isothermal DNA amplification assays do not need a controlled thermal cycling complex device. Moreover, these assays offer greater utility in the field by including simplistic reactor designs or portable heat sources [13]. Recombinase polymerase amplification (RPA) is an isothermal technology, which amplifies DNA at a constant temperature between 25°C and 42°C [13]. RPA depends on a combination of recombinase, single strand binding protein and strand displacing DNA polymerase for the DNA amplification step. Real-time detection of RPA amplicons relies on the exonuclease enzyme, which cuts at the basic mimic site presence between fluorophore and quencher in the RPA exo-probe. The RPA is extremely fast (3-15 minutes) and all reagents are cold chain independent. There have been developed several RPA assays to detect a wide range of different pathogens such as group B streptococci, *Brucella* or *Mycobacterium tuberculosis* [13-15].

In this study, a real-time RPA assay detecting MAP-DNA was developed. The assay sensitivity, specificity and cross reactivity were determined. The clinical performance of the MAP RPA assay was evaluated by 48 MAP-positive and 20 MAP-negative blood, sperm, faecal and tissue samples. All results were compared by a well-established real-time PCR [16].

## **Materials and Methods**

### ***Ethical statement***

In total, the study included 68 archived DNA samples, which have been collected during routine veterinary examination in the Institute of Veterinary Medicine, Goettingen. All samples were taken under consideration of the German codex “Gute Veterinärmedizinische Praxis”.

### ***Generation of a molecular and genomic DNA MAP Standards by PCR***

MAP (ATCC 19698) was ordered from Leibniz Institute DSMZ-German Collection of Microorganisms and cell cultures (DSMZ, Braunschweig, Germany, ID: 44133). DNA was extracted as follows: after culturing MAP on Herrold's Egg Yolk Agar Slants medium containing mycobactin and amphotericin B, nalidixic acid, vancomycin (BD, Franklin Lakes, NJ, USA) for 5 weeks, pure colonies were suspended in 100 µl water and incubated at 99°C to 100°C with shaking at 300 rpm in a Bioer Mixing Block MB-102 (Bioer Technology, Hangzhou, China). After 20 minutes, the suspension was centrifuged at 14000 g for 10 minutes. Then the supernatant was collected and centrifuged at 14000 g for 10 minutes. The amount of DNA in the supernatant was measured by a NanoDrop ND-1000 spectrometer (Thermo Scientific, Waltham, MA, USA). A genomic DNA standard containing 10 ng/µl to 10 fg/µl of DNA dissolved in water was prepared to test the analytical sensitivity of the RPA assay. To generate the plasmid standard, a 587 bp fragment was amplified covering the nucleotides 183 to 769 of MAP Gene IS900 sequence (Genbank accession number: AF416985.1) using the published primer sequences 5'-GTCGGCGTGGTCGTCTGCTGGGTTGAT-3 as a forward primer (FP) and 5'-GCGCGGCACGGCTCTTGTTGTAGTC-3 as a reverse primer (RP) [17]. The PCR reaction was performed on a T3000 thermocycler (Biometra GmbH, Goettingen, Germany) using the following reaction mix: 11.8 µl molecular

biology H<sub>2</sub>O, 2 µl BSA (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 2 µl 10x Standard Reaction Buffer containing MgCl<sub>2</sub> (Biotools B&M Labs. S.A., Madrid, Spain), 0.4 µl DMSO (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 0.4 µl of each primer (10 µM stock solution), 0.1 µl Biotools DNA polymerase (Biotools B&M Labs. S.A., Madrid, Spain), 0.1 µl of each of the 10 µM aNTP, tNTP, cNTP and gNTP stock solutions (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 2.5 µl of the template. The PCR cycles were programmed as follows: initial denaturation step at 95°C for three minutes, then thirty cycles of 95°C/30 sec, 64°C/60 sec and 72°C/60 sec and final extension step at 72°C for eight minutes. The amplified fragment was then cloned using the pGEM-T Easy Vector Systems I kit (Promega, Madison, USA) according the manufacture instructions. The number of plasmids were calculated using an equation as previously described [18]. The plasmids were linearized using the NcoI-HF (New England Biolabs, Ipswich, MA, USA).

A dilution range of 10<sup>0</sup> to 10<sup>6</sup> molecules/µl of the standard was prepared. Both the molecular plasmid and genomic DNA standard were tested using a published quantitative real-time PCR protocol ([16], patent number: EP2841596A1) as described below.

### **Real-time PCR**

The real-time PCR assay was performed on a LightCycler 480 (Roche, Mannheim, Germany) using the LightCycler 480 Probes Master Kit (Roche, Mannheim, Germany) to amplify a 139 bp long fragment (nucleotides 523 to 636 of the Genbank accession number: AF416985.1). The Reaction volume contained 10 µl Light Cyclor 480 Probes Master mix, 0.5 µl of each 10 µM FP: 5'-TACCGCGGCGAAGGCAAGAC-3' and RP: 5'-CGGAACGTCGGCTGGTCAGG-3', 1 µl of 10 µM probe: 5'-FAM-ATGACATCGCAGTCGAGCTG-BHQ-1-3 and 3 µl molecular biology H<sub>2</sub>O as well as 5 µl of the DNA template. The LightCycler was programmed as follow: first ten minutes pre-incubation at 95°C, then 45 cycles of 95°C/15 sec, 60°C/30 sec and 72°C/35 sec followed by a final cooling step at 40°C for 30 sec.

### ***Real-time RPA primers and exo-probes***

Three regions (RPA1, RPA2 and RPA3) flanking the IS900 gene sequence were tested to select an area generating the highest RPA assay sensitivity (S1 Fig). In total, 18 forward primers, 25 reverse primers and three exo-probes were tested. The exo-probe

was produced by TIB MOLBIOL (Berlin, Germany), while all the primers were purchased from Eurofins MWG Synthesis GmbH (Ebersberg, Germany). RPA primers were purified by gel filtration and the exo-probe by HPLC.

#### ***RPA assay conditions***

The MAP RPA assay was performed using the TwistAmp Exo “Improved Formulation” kit (TwistDx Ltd, Cambridge, UK) according to the manual instruction. Briefly, 29.5 µl Rehydration buffer, 6.7 µl H<sub>2</sub>O, 2.1 µl of 10 µM of both FP and RP, 0.6 µl of the 10 µM of the exo-probe and 5 µl of the DNA template were added to a freeze dried reaction pellet. For the negative control water was used instead of the DNA template. The RPA reactions were incubated at 42°C for fifteen minutes using a Tubescanner (Twista, TwistDx Ltd, Cambridge, UK). A mixing step by vortexing was performed after 230 seconds of the incubation in order to improve the sensitivity of the assay. The FAM fluorescence signal intensities were visualized on the Tubescanner studio software (version 2.07.06, QIAGEN Lake Constance GmbH, Stockach, Germany). For signal interpretation, threshold and first derivative analysis were applied.

#### ***Analytical Sensitivity and specificity***

To determine the analytical sensitivity of the RPA assay, the molecular standard ( $10^6$  to  $10^0$  molecules/µl) was tested eight times and the genomic DNA standard (10 fg/µl to 10 ng/µl) in triplicate in both RPA and real-time PCR. The threshold times and the CT values were plotted against the number of molecules detected. Semi-log non-regression analysis was calculated with PRISM (Graphpad Software Inc., San Diego, California) and probit analysis was performed by STATISTICA (StatSoft, Hamburg, Germany). The analytical specificity of the RPA assay was tested with DNA isolated from different bacteria strains listed in Table 1. All bacteria species except *Mycobacterium bovis* were provided by DSMZ and grown on media following the instructions given by the DSMZ. All mycobacteria were tested positive with 16S ribosomal DNA PCR assay to ensure DNA extraction quality [19]. *Mycobacterium bovis* DNA (ATCC: 27289) was provided by Institute for molecular Pathogenesis, Friedrich Loeffler Institut, Federal Research Institute for Animal Health, Jena, Germany.



**Table 1:** List of bacteria species and strains used for determining the cross reactivity of the RPA assay.

<b>Name of Bacteria</b>	<b>DSMZ ID</b>
<i>Mycobacterium bovis</i>	N/A
<i>Mycobacterium avium</i> subsp. <i>avium</i>	44156
<i>Mycobacterium kansasii</i>	44162
<i>Mycobacterium marinum</i>	44344
<i>Mycobacterium fortuitum</i>	46621
<i>Mycobacterium avium</i> subsp. <i>silvaticum</i>	44175
<i>Mycobacterium avium</i> <i>intracellulare</i>	43223
<i>Mycobacterium phlei</i>	43239
<i>Mycobacterium goodii</i>	43213
<i>Mycobacterium smegmatis</i>	43756
<i>Mycobacterium scrofulaceum</i>	43992
<i>Rhodococcus hoagii</i>	20307
<i>Escherichia coli</i>	30083
<i>Clostridium perfringens</i>	756
<i>Staphylococcus aureus</i>	799
<i>Listeria monocytogenes</i>	15675
<i>Streptococcus uberis</i>	20569
<i>Streptococcus agalactiae</i>	2134
<i>Enterococcus faecalis</i>	1103

### ***Clinical samples***

Archived DNA of MAP positive blood (n=14), sperm (n=18), faecal (n=12) and tissue (n=4) samples as well as 20 MAP-negative faecal samples were tested in both RPA and real-time PCR in triplicate as described above. DNA extraction was performed by QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) using the recommended modifications as described previously [16].

## Results

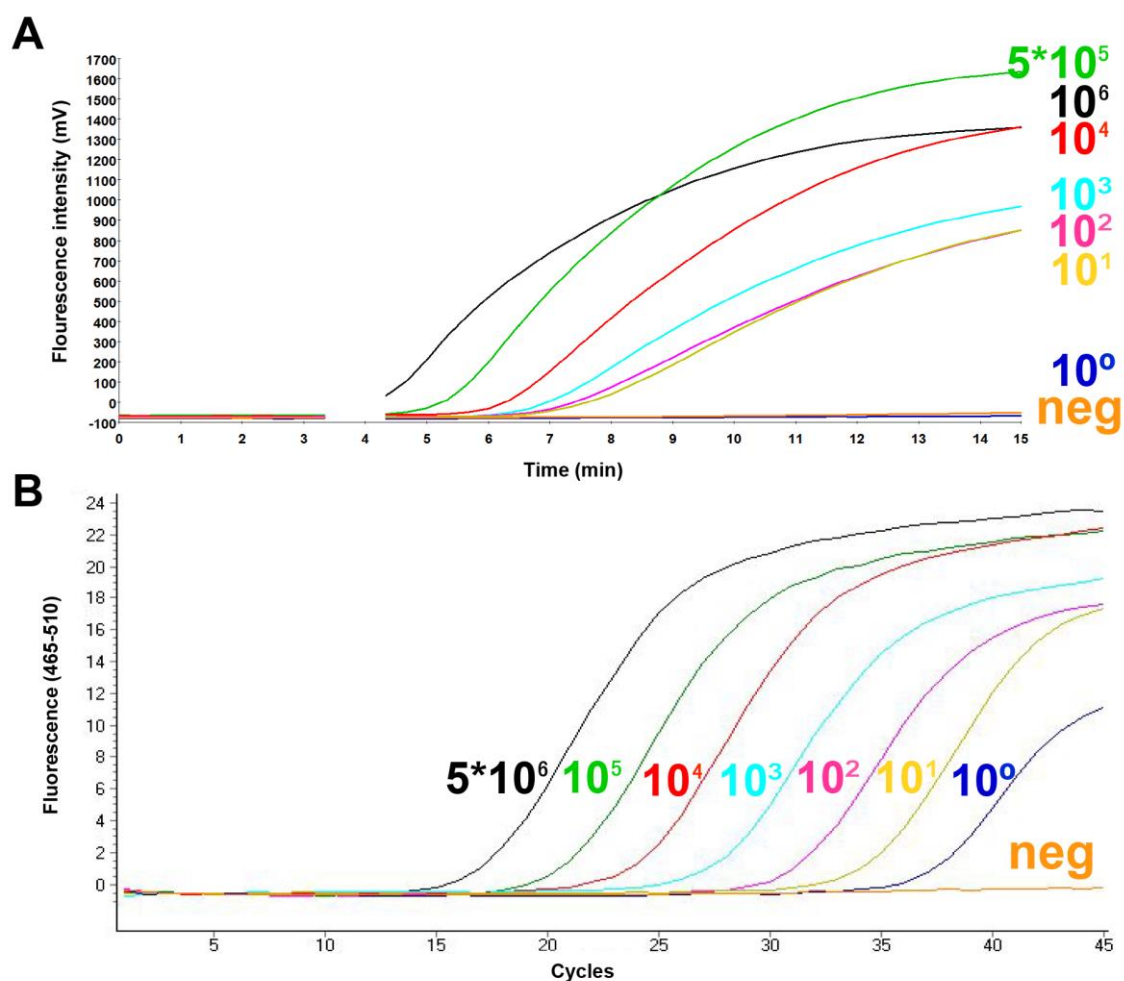
To determine the analytical sensitivity, a dilution range of the molecular DNA MAP standard was tested in RPA using the primer-probe combinations for three different regions in the IS900 gene sequence of the MAP (S1 Fig). Both RPA1 and RPA2 assays did not produce exponential amplification curves. Start points of the fluorescence signal for  $5 \times 10^5$  standard DNA were eight and seven minutes, respectively (S2 Fig). In contrast, for the primer combination FP1+RP2+P3 from RPA3 assay, the start point was between four and five minutes and the detected concentration of molecular plasmid standard was down to 50 DNA copies (Fig 1, Table 2). This combination was used for the further validation of the RPA assay. The data set of eight replicates of the molecular DNA standards of both RPA and real-time PCR assays was used in the semi-log and probit analyses. The time needed to reach the detection limit was below ten minutes in the RPA assay and 90 minutes in the real-time PCR assay (Fig 2). In probit analysis, the limits of detection with a 95% probability were 16 and one molecules of MAP plasmid standard DNA per microliter in RPA and real-time PCR assays, respectively (Fig 3). Using the genomic DNA, the limit of detection for RPA and real-time PCR assays were 500 fg/reaction and 50 fg/reaction, respectively (Fig. 4).

The MAP RPA assay was highly specific, as it did not detect the DNA of other mycobacteria and environmental bacteria listed in Table 1. In addition, twenty MAP negative faecal samples were negative in both RPA and real-time PCR. Out of 48 real-time PCR positive DNA samples, forty-three were positive for MAP detection in RPA (89.5%, S1 Table).

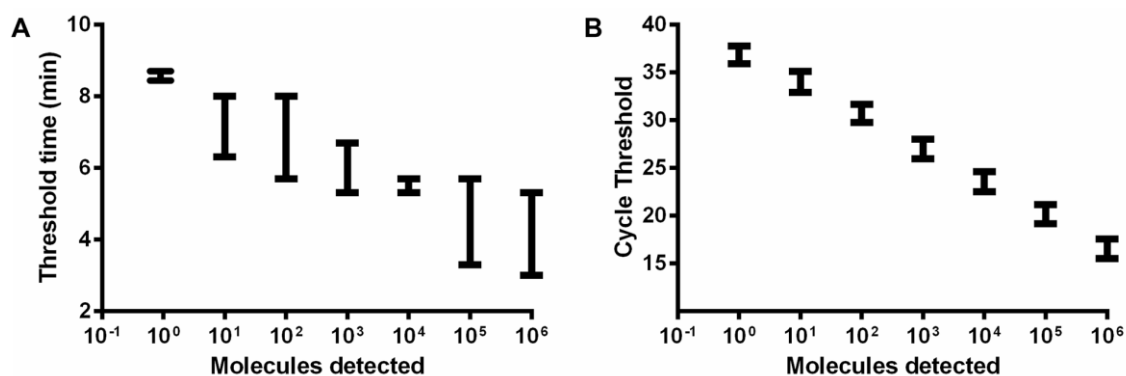
**Table 2:** RPA primers and exo-probe combination, yielding the highest analytical sensitivity in the MAP-RPA assay.

Name	Sequence (5' to 3')
Probe.RPA3	ACGCCGGTAAGGCCGACCATTACTGCATGGTQTFTAACGA CGACGCGCA
MAP.RPA3.FP1	CGTGGACGCCGGTAAGGCCGACCATTACTGCATGG
MAP.RPA3.RP2	CGCCGCAATCAACTCCAGCAGCGCGGCCTC

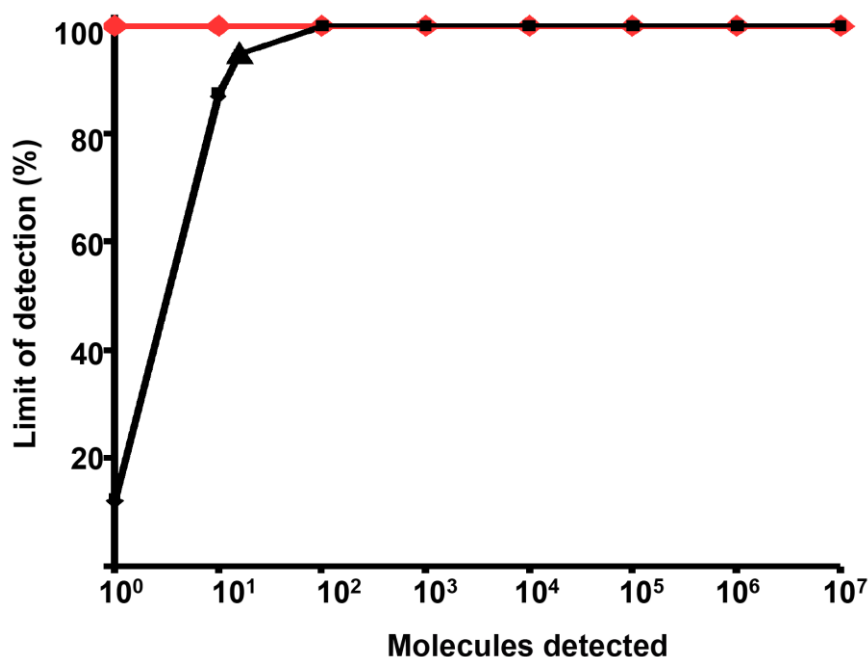
QTF are sites of the quencher and fluorophore in the order quencher BHQ1-dt (Q), Tetrahydrofuran (T) and Fam-dT (F).



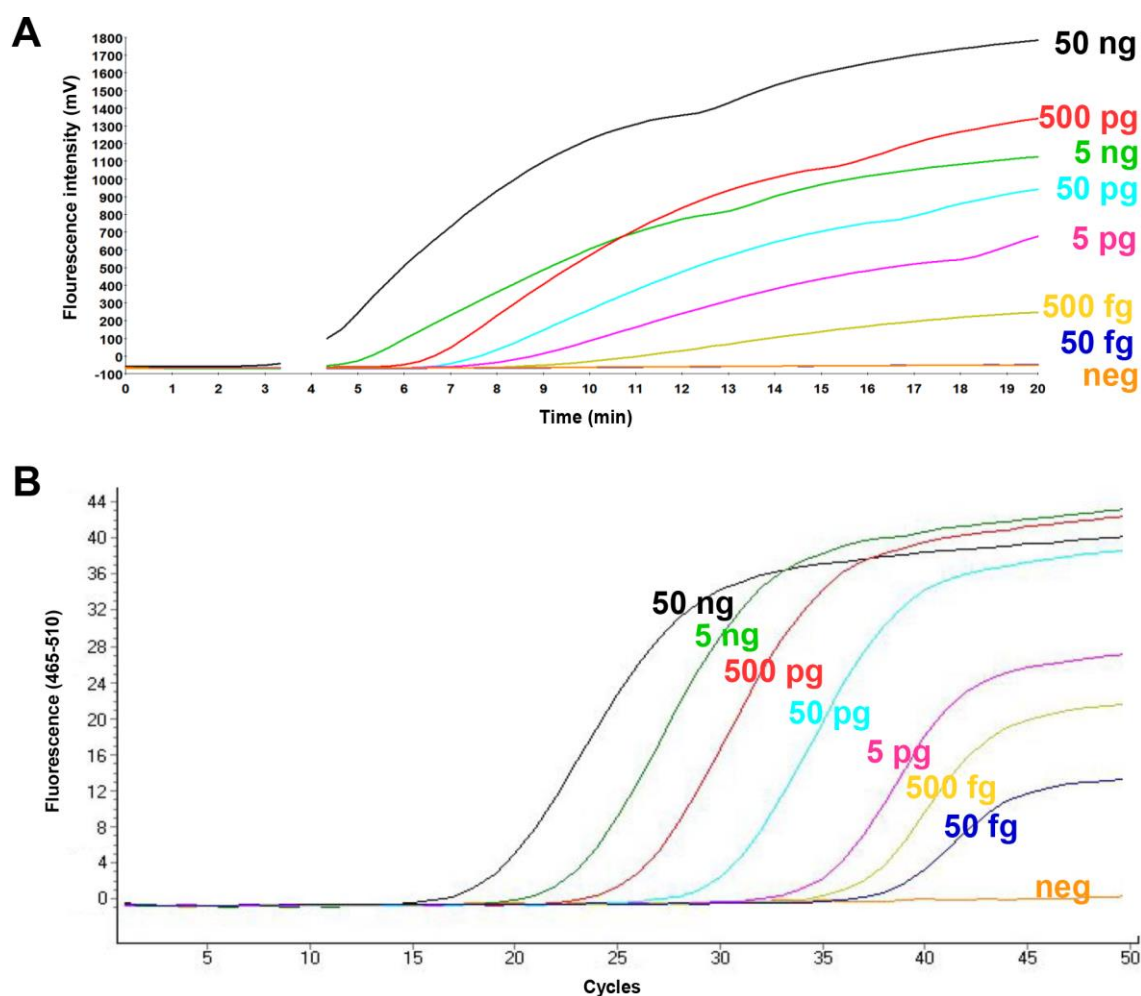
**Fig. 1:** Amplification curves in MAP RPA (A) and MAP real-time PCR (B) assays applying serial dilutions ( $5 \times 10^6$ -1) of the molecular plasmid DNA standard. Analytical sensitivity of RPA and real-time PCR assays were 50 and 5 molecules/reaction, respectively. No fluorescent signals were detected in RPA after three minutes because the strip was taken out for mixing.



**Fig. 2:** Reproducibility of the RPA (A) and real-time PCR (B) assays using data set of eight runs of serial dilution of the MAP plasmid molecular standard in PRISM. RPA assay produced results between 2 to 10 minutes. In RPA  $5 \times 10^6$ - $10^2$  DNA molecules were detected in 8 out of 8 runs; 50, 7/8 and 5, 1/8 by the RPA assay. The error bars represent the range. The real-time PCR produced more linear results, due to the regular cycle format of the PCR, while there is no strict separation of the amplification cycles in the isothermal RPA technology.



**Fig. 3:** Probit analysis results for the MAP RPA (black) and real-time PCR (orange) assays using STATISTICA. Data sets of eight RPA and real-time PCR assay runs as showed in Fig. 1 was used. The limit of detection in RPA and real-time PCR at 95% probability were 16 and one DNA molecules of the molecule plasmid DNA standard, respectively.



**Fig. 4:** Amplification curves in RPA (A) and real-time PCR (B) using the genomic MAP DNA standard isolated from MAP (ATCC 19698). The amount of the DNA in each dilution was calculated using NanoDrop. RPA detected down to 500 fg DNA/reaction, while real-time PCR identified 50 fg DNA/reaction.

## Discussion

In this study, we have developed a rapid RPA test detecting the IS900 gene sequence of MAP. The limit of detection using a molecular standard was 16 DNA molecules and 500 fg by employing the genomic DNA. The latter corresponds to approximately 95 MAP genomes as one MAP genome has a weight of 5.29 fg [16].

Three regions of the IS900 gene (RPA1, RPA2, RPA3, see S1 Fig) were tested in our setup. The RPA3 superposed the RPA1 and RPA2 assays in producing an exponential amplification curve and early fluorescence signal. The GC contents of RPA1, RPA2 and

RPA3 regions were 70% and 67% and 55%, respectively. The higher GC content of the target gene is able to slow down the performance of the strand exchanging proteins in the reaction [20, 21], which may explain the higher sensitivity of the RPA3 region containing lower GC content.

The clinical sensitivity of our assay was around 89.5% in comparison to real-time PCR upon using archived frozen DNA extracts. Although RPA is resistant to most PCR inhibitors [22], the presence of high amount of background DNA has a negative influence on the RPA sensitivity [23]. In contrast, false negative samples in our assay contain lower amount of total DNA than positive sample as determined by Nanodrop (S1 Table). The decrease in assay sensitivity due to low MAP DNA content in the sample was also excluded as two of the false negative samples had a high content of MAP DNA as determined by real-time PCR (CT: 26-29). In addition, the RPA assay detected four samples of high CT values (between 34 to 35).

Another point of need method named Loop-mediated isothermal amplification (LAMP) was applied to detect MAP DNA [24]. The LAMP assay turnout time was 100 minutes to detect 100 fg of MAP genome. Moreover, six primers were employed to amplify the respective MAP gene. The results readout relies on the changes in the assay turbidity, which can be recognized by the naked eye or recorded in real-time by a portable device. In contrast, our RPA assay required 15 minutes process time, utilizes two primer and implemented a probe system to increase the assay specificity.

The MAP RPA assay was developed for the rapid and accurate detection of MAP DNA. A possible use of the RPA assay in combination with a fast DNA extraction protocol will allow its application for detection of MAP directly in the field.

**List of Abbreviations**

BHQ1	Black Hole Quencher 1
bp	Base pair
BSA	Bovine serum albumin
CT	Cycle threshold
dATP	Desoxyadenosintriphosphat
dCTP	Desoxycytidintriphosphat
dGTP	Desoxyguanosintriphosphat
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
dTTP	Desoxythymidintriphosphat
ELISA	Enzyme-linked Immunosorbent Assay
FAM	6-carboxyfluorescein
fg	Femto gram
Fig	Figure
FP	Forward primer
g	G-Force
H <sub>2</sub> O	Water
LAMP	Loop mediated isothermal amplification
MAP	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>
MgCl <sub>2</sub>	Magnesium dichloride
N/A	Not available
ng	Nano gram
PCR	Polymerase Chain Reaction
P	Probe
pg	Pico gram
RPA	Recombinase Polymerase Amplification
RP	Reverse primer
rpm	Rounds per minute
sec	Second
TT	Time threshold
µl	Micro liter



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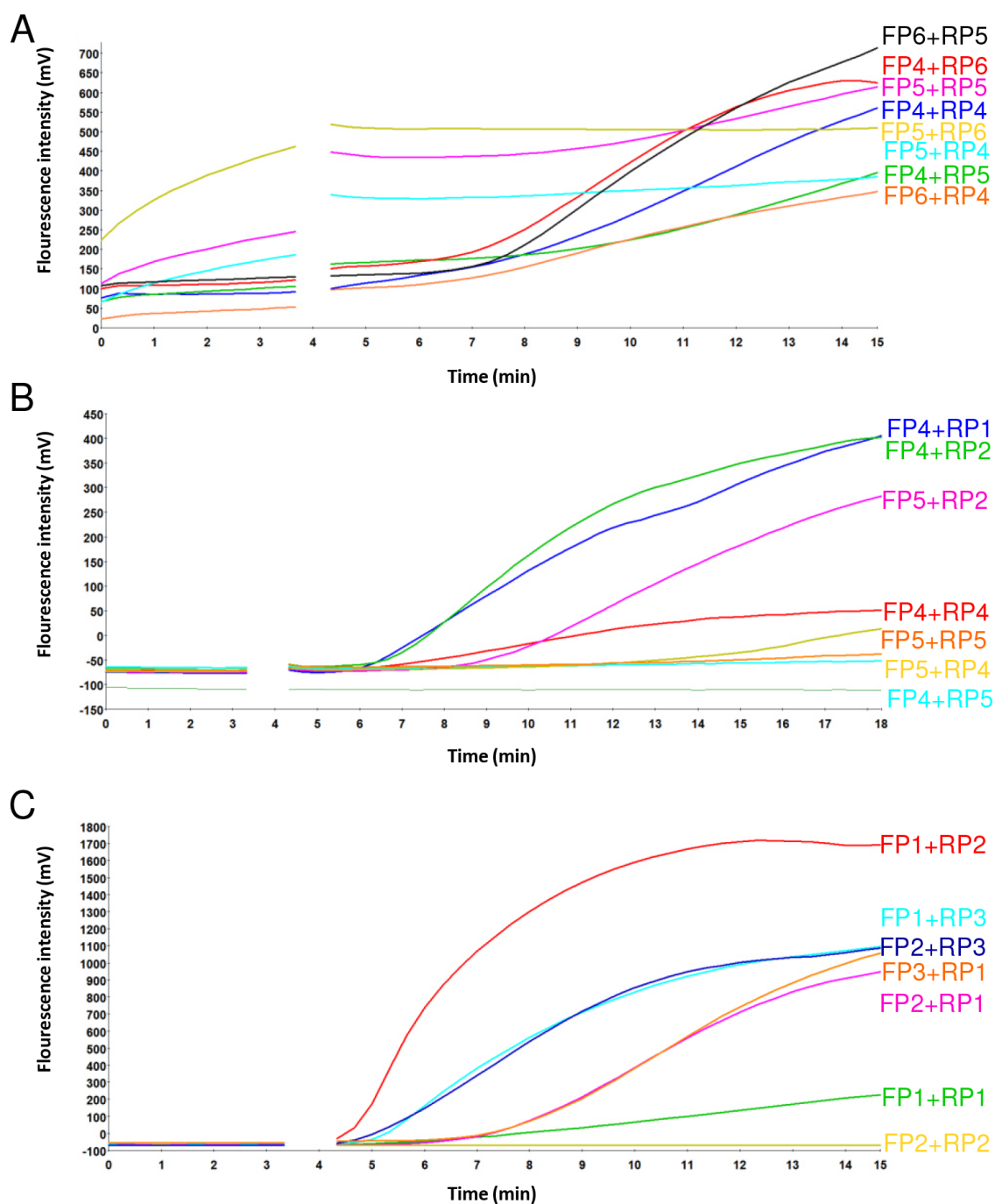
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**S1 Fig. MAP RPA 1-3 primer and probe sequences.** (A) MAP-RPA 1 (nucleotides 483-769; Genbank accession number: AF416985.1): Nine forward primers and eight reverse primers were tested. (B) MAP-RPA 2 (nucleotides 353-645; Genbank accession number: AF416985.1): Six forward primers and fourteen reverse primers were tested. (C) MAP-RPA 3 (nucleotides 182-405; Genbank accession number: AF416985.1): Three forward primers and three reverse primers were tested. Q1F are sites of the quencher and fluorophore in the order quencher BHQ1-dt (Q), Tetrahydrofuran (T) and Fam-dT (F). RC is the reverse complementary of the original sequence used in the experiment.



**S2 Fig.** Amplification curves of screening primers combinations targeting three RPA regions on the IS900 gene. A, RPA1; B, RPA2; C, RPA3. RPA3 FP1+RP2 produced the best exponential curve and a start point after 4 minutes.

**S1 Table.** Results of screening MAP-positive DNA samples using both real-time PCR and RPA assays. All results are mean out of triplicate. CT is the cycle threshold and TT is the threshold time. The amount of DNA in each sample was calculated using NanoDrop.

Sample ID	Matrix	Real time PCR (CT)	RPA (TT)	NanoDrop (ng/μl)
1	feces	30.74	6	15,11
2	blood	32.81	10,5	40,34
4	feces	32.24	6,5	11,22
5	feces	30,00	6	21,95
6	blood	32,25	6	3,92
7	sperm	34,81	neg	33,43
8	sperm	30,04	8.0	52,46
9	feces	30,81	8.0	25,38
10	blood	34,13	9.5	1,52
11	blood	30,12	7.0	13,48
12	sperm	29,92	8.0	19,12
13	sperm	32,38	7.25	53,73
14	blood	36,85	neg	12,49
15	blood	32,48	7.5	10,49
16	sperm	34,88	11.5	7,23
17	sperm	35,02	10.6	14,68
18	feces	33,42	7.0	98,92
20	blood	33,24	6.0	25,28
21	blood	29,93	7,5	33,95
22	blood	28,92	5.5	6,03
23	blood	29,28	6.0	0,64
24	blood	28,92	6.5	1,99
25	sperm	29,40	10.0	176,52
26	sperm	28,92	8.0	148,90
27	blood	31,52	6.5	7,68
28	sperm	31,77	5.35	186,96
29	sperm	32,05	4.65	175,92
30	sperm	30,70	12.0	147,51
31	sperm	29,90	neg	149,33
32	feces	32,83	9.25	78,98
33	sperm	26,93	neg	183,44
34	sperm	27,33	3.5	511,83
35	feces	22,56	14.25	92,72
36	feces	22,94	5.5	62,11
37	feces	19,03	4.75	75,88
38	feces	23,55	5.0	56,11
39	blood	20,58	11.0	11,53
40	blood	24,41	11.0	12,52
41	tissue goat	26,77	7.25	299,18

**S1 Table.** continued

<b>Sample ID</b>	<b>Matrix</b>	<b>Real time PCR (CT)</b>	<b>RPA (TT)</b>	<b>NanoDrop (ng/μl)</b>
<b>42</b>	tissue goat	22,65	3.5	235,80
<b>43</b>	tissue goat	31,73	14,50	98,62
<b>44</b>	tissue goat	33,91	8,00	79,57
<b>45</b>	sperm	32,63	9.65	414,81
<b>46</b>	sperm	32,16	7.5	385,90
<b>47</b>	sperm	32,01	neg	286,59
<b>48</b>	sperm	32,14	10,00	250,48
<b>49</b>	feces	35,26	6,00	130,73
<b>50</b>	feces	27,05	7,00	63,70



## AUTHOR'S CONTRIBUTIONS

### **Chapter I: Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in a Subclinical Naturally Infected German Fleckvieh Bull**

The author (KF) established the matrix-specific adaptation for DNA purification und cultural preparation, investigated faecal, semen, blood and tissue samples, performed sequencing, analysed the sequences, and was the primary writer of the manuscript.

### **Chapter II: Detection of *Mycobacterium avium* subsp. *paratuberculosis* in non-human primates**

The author (KF) established the matrix-specific adaptation for DNA purification und culture preparation, investigated faecal and tissue samples, performed sequencing, analysed the sequences, and was the primary writer of the manuscript.

### **Chapter III: Detection of *Mycobacterium avium* subsp. *paratuberculosis* in captured wildlife rock hyraxes (*Procavia capensis*)**

The author (KF) analysed all results of the PCR, culture and ELISA experiments, and was the primary writer of the manuscript.

### **Chapter IV: Development of a recombinase polymerase amplification assay for rapid detection of the *Mycobacterium avium* subsp. *Paratuberculosis***

The author (KF) supported the data analyses and the scientific writing process.

### **3 GENERAL DISCUSSION**

## DISCUSSION

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the aetiological agent of paratuberculosis, a disease found worldwide that mainly affects ruminants. In the agricultural sector, this disease causes significant financial losses, especially for the dairy industry. Furthermore, the involvement of MAP in the pathological process of Crohn's disease in humans has been controversially discussed for many years. It is assumed that the consumption of animal products such as milk, cheese, and meat are possible vectors of MAP transmission from animals to humans. The potential zoonotic risk and the economic losses in the agricultural sector are two main reasons for the indispensability of effective control of the pathogen. The long incubation period of paratuberculosis without visible clinical signs leads to the unrecognized spread of the bacteria. Unfortunately, the current diagnostic possibilities are limited, especially during the subclinical stage of the disease. In particular, an incomplete understanding of the pathogenesis and immunology during this stage of the disease leads to diagnostic uncertainties.

### *Detection pattern of MAP in a subclinically infected bull*

This thesis (**chapter I**) provides detailed information about the occurrence of MAP in a naturally infected, but clinically unremarkable, bull from the age of seven weeks until necropsy (a period of four years). In the longitudinal study performed, the detectability of MAP in faeces, semen, and blood was periodically investigated by PCR and bacterial culture. The immune response against MAP was measured by a commercially available antibody ELISA. The results of this study clearly demonstrate the difficulties involved in MAP diagnosis during the subclinical stage of the disease. MAP was only intermittently present in one, two or all three investigated matrices. On 65% of the sample days (up to nine weeks), the pathogen was undetectable by both PCR and bacterial culture. The detectability of the pathogen was not predictable by either PCR or bacterial culture. As outlined in the general introduction (Section: Control measures in Europe), most control programs in Europe are based on the detection of an antibody response against MAP in milk or blood by commercially available ELISA. This method is used due to the low costs and the feasibility of investigating large sample numbers. However, although the bull excreted MAP in concentrations of up to  $6.25 \times 10^6$  genome

equivalents/g faeces, an antibody response against MAP was not measured during the entire investigation period. Animals without a measurable antibody response spread the pathogen unrecognized within and between the herd, and may be one reason for the reduced success of paratuberculosis control programs. In agreement with our results, a recent study showed that unpredictable monthly variations exist in the test results of cows using a commercial milk ELISA, solid and broth faecal culture, and faecal real-time PCR (Laurin et al., 2017). To date, the exact mechanisms involved in the pathogenesis and immune response during the subclinical stage of the disease remain incomplete. The resulting reduced efficiency of diagnostic assays leads to the unrecognized spread of MAP. Therefore, the development of an efficient diagnostic strategy to identify subclinically infected animals should remain a priority.

#### ***Distribution of MAP within subclinically infected animals***

Clinical symptoms develop in only 10-15% of MAP-infected cattle within one to ten years after infection (Olsen et al., 2002). Most of the infected animals remain in the subclinical disease stage. Animals in this stage do not show clinical signs; however, they do intermittently excrete the pathogen (Olsen et al., 2002). This investigation (**chapter I**) was conducted to provide detailed information about the distribution of MAP within a naturally infected, but clinically unremarkable bull. The examination of the collected tissues demonstrated that MAP can be detected by PCR in tissues of the lymphatic system (7/15), digestive tract (5/14), and the urogenital tract (5/9), with concentrations of up to  $3.9 \times 10^6$  MAP-GE/g tissue. Although MAP was widely spread within the host, neither macroscopic nor histological lesions, characteristic of a MAP infection, were observed. In general, such a bland pathological picture in PCR-positive samples is an uncommon finding in MAP-infected ruminants. However, another study also reported no macroscopic lesions or clinical signs after confirming the presence of MAP in many tissues of the cattle (Brady et al., 2008). Therefore, it can be assumed that MAP can replicate within the asymptomatic host without triggering pathological changes. While paratuberculosis in domestic ruminants is intensively researched, little is known about the clinical signs and/or pathological and histological changes of MAP infection in non-ruminants. MAP has previously been detected in many non-ruminant animal species, such as foxes (*Vulpes vulpes*), stoats (*Mustela erminea*), weasels (*Mustela nivalis*), crows (*Corvus corone*), and wood mice (*Apodemus sylvaticus*) (Beard

et al., 2001a; Carta et al., 2013; Greig et al., 1999). Although the pathogen has been found in these animals, no or only mild gross pathological changes and histopathological lesions were often found. In the present investigation (**chapters II and III**), MAP was detected in the tissues of rock hyraxes (*Procavia capensis*) and in two non-human primate species (cottontop tamarin, *Saguinus oedipus* and common marmoset, *Callithrix jacchus*). The pathogen was detected in these animals by PCR, but typical clinical signs, and macroscopic or microscopic pathological changes were not observed. As demonstrated in the investigated bull, MAP is replicated and excreted via faeces without triggering typical macroscopic or microscopic changes (**chapter I**). It could therefore be assumed that MAP may also replicate in non-ruminants without eliciting characteristic lesions. Therefore, infected non-ruminants should also be considered a source of pathogen spread.

It was striking that MAP DNA was detected in the bone marrow of the German Fleckvieh bull (**chapter I**) and the common marmoset (**chapter II**). MAP was detected in only one previous study in the bone marrow of two stump-tail macaques (*Macaca arctoides*) by microscopic examination (McClure et al., 1987). A recent study indicated that a MAP-related pathogen, *Mycobacterium tuberculosis*, persists within the CD271<sup>+</sup>/CD45<sup>-</sup> mesenchymal stem cells of the bone marrow in a viable but non-culturable (VBNC) state (Das et al., 2013). The VBNC state is assumed to be a potential survival strategy of MAP (Lamont et al., 2012; Whittington et al., 2004). New data suggest that sporulation-like mechanisms may be a strategy used by MAP to accomplish persistence in the host and the surrounding environment (Lamont et al., 2012). VBNC bacteria likely adapt to less optimal living and reproduction conditions through a wide range of metabolic modifications, including changes in the cell wall, or down-regulation of DNA replication or cell division (Lamont et al., 2012; Mukamolova et al., 2003; Rowe and Grant, 2006; Whittington et al., 2004). Although bacteria in the VBNC state fail to grow by standard bacteriological methods, they remain alive (Oliver, 2010). Furthermore, mesenchymal stem cells found in bone marrow appear to play a key role in the establishment of latent tuberculosis (Das et al., 2013; Raghuvanshi et al., 2010). Hence, the bone marrow may also provide a niche for latent infections with MAP in ruminants as well as non-ruminants. To investigate the occurrence of MAP in the bone marrow, further studies should include an examination of MAP in this tissue, in addition to gastrointestinal tissues and lymph nodes.

*Transmission of MAP via the faeces of calves*

For the effective control of paratuberculosis, it is necessary to consider all possible routes of transmission. A central factor in the transmission of MAP is the infection of newborn calves. These animals are usually infected shortly after their birth via the faecal-oral route. Colostrum and milk of subclinically (Streeter et al., 1995) as well as clinically infected cattle (Taylor et al., 1981) can contain the pathogen, but this is usually in low numbers (Grant et al., 2001). Faecally contaminated colostrum, milk and food are the main sources of infection. In addition to asymptomatic, infected adult animals, experimentally infected calves younger than eight months also sporadically excrete MAP in faeces (Collins and Zhao, 1994; McDonald et al., 1999). The results of the present study (**chapter I**) confirm the repeated excretion of MAP in a naturally infected calf between the ages of seven and 16 weeks. Over the entire investigation period (four years), the highest MAP genome equivalent concentration ( $6.25 \times 10^6/\text{g}$ ) was measured in the faeces of the bull calf. Such high concentrations of MAP may be high enough to elicit an infection in another susceptible animal, as a concentration of  $10^3$  MAP/animal is estimated to be sufficient to infect calves (Brotherston et al., 1961). However, measuring the concentration of MAP based on the results of the performed real-time PCR and a differentiation between viable and dead MAP cells is not possible with this method. Furthermore, the differentiation between a true infection (active MAP shedding) and a pass through (passive MAP shedding) of MAP is difficult to ascertain, especially for calves (van Roermund et al., 2007). True infection of the investigated bull calf was confirmed at the age of 40 weeks by initial detection of MAP DNA in the blood. The detection of MAP within the blood indicates that the pathogen crosses the intestinal mucosa, establishing a true infection. The possible risk of calves as a source of infection underlines the importance of immediate separation of calf and cow after birth. This separation is one of the key measures that will prevent the transmission of MAP from cow to calf and in turn the transmission from calf to calf (Kirkeby et al., 2016), whether they are truly infected or simply pass through the pathogen.

*Sexual transmission of MAP*

In addition to the faecal-oral infection, it is well known that MAP can be transmitted vertically from subclinically as well as clinically infected cows *in utero* to the foetus

(Pribylova et al., 2013). However, the role of bulls in the spread of MAP has been poorly explored. Since 1970, it has been known that MAP is detectable in the reproductive organs and semen of bulls (Larsen and Kopecky, 1970). To date, only a few studies have been performed on the occurrence of MAP in bulls. The results of all performed studies were listed in Table 7 and 8, demonstrating the occurrence of MAP in bull reproductive organs and semen. Nevertheless, the role of bulls in the vertical transmission of MAP via semen remains unanswered.

Therefore, another objective of the present thesis (**chapter I**) was to monitor the excretion of MAP-contaminated semen and the distribution of MAP within the male reproductive organs of an infected, but asymptomatic bovine host. MAP DNA was detected in only four of the 89 semen samples (5%) by snPCR. This failure of detection and/or quantification by qPCR and bacterial culture indicated the presence of only low numbers of MAP. In general, it has been argued that the transmission of MAP is unlikely, due to low numbers of MAP in semen (Buergelt and Williams, 2004; Philpott, 1993). However, in a longitudinal study of another asymptotically infected Fleckvieh bull, MAP DNA was repeatedly detectable in concentrations of up to  $5.7 \times 10^5$  MAP genome equivalents/ml (Münster et al., 2013b). It is not clear if such concentrations of MAP in semen are enough to elicit an infection. In addition to the monitoring of MAP contamination in semen, the presence of MAP within the male reproductive organs was screened in this investigation (**chapter I**). In the present study, the pathogen was detected in five of nine urogenital tract tissue samples, including the testicle, epididymis, ampulla of deferent duct, prostate gland, and kidney. In contrast to the investigated bull, all other previously examined bulls showed clinical symptoms, or if they were asymptomatic, typical macroscopic abnormalities were found during necropsy, indicating an advanced disease stage (Ayele et al., 2004; Glawischnig et al., 2004; Khol et al., 2010; Larsen and Kopecky, 1970; Larsen et al., 1981). The detection of MAP in the semen as well as in the reproductive organs supports the hypothesis that asymptomatic bulls likely transmit MAP via semen, especially under natural mating conditions. In cases of artificial insemination, the influence of antibiotics in various semen extenders as well as the freezing process on the viability of MAP in semen should be tested. To monitor the occurrence of viable MAP in native and diluted semen of subclinically as well as clinically infected breeding bulls, the development of a sensitive and specific method is necessary. One promising method for the detection of

viable MAP may be a phage-based assay, due to a less time-consuming detection in comparison to culture-based approaches. The phage assay has been adapted for the detection of MAP in many different matrices, such as milk and blood (Botsaris et al., 2016; Foddai et al., 2011; Foddai and Grant, 2017; Stanley et al., 2007; Swift et al., 2013); however, it has not yet been adapted for the detection of MAP in semen.

Surprisingly, MAP was also detectable by PCR in the reproductive organs of one female and one male rock hyrax (**chapter III**). The renewed detection of MAP within the reproductive organs of rock hyraxes (**chapter III**) supports the hypotheses that MAP may be sexually transmitted via bull semen (**chapter I**). To the best of the author's knowledge, this is the second report on the presence of MAP within the reproductive organs of naturally infected non-ruminant wildlife species. Primarily, MAP was detected in the reproduction organs of rabbits (*Oryctolagus cuniculus*) from Scotland by PCR and bacterial culture (Judge et al., 2006). In contrast to cattle (syndesmochorial placenta), rabbits as well as rock hyraxes have a haemoendothelial placenta (Telugu and Green, 2008). In this placenta type, the uterine epithelium, the chorionic villi, and the chorionic mesenchyme are absent. Thus, embryonic capillaries are directly located in the maternal blood. Due to these anatomical characteristics, the vertical transmission of MAP *in utero* is much more likely in rabbits (Telugu and Green, 2008) and rock hyraxes than in cattle. Further studies are needed to investigate the sexual route of transmission in non-ruminant hosts.



**Table 7:** Summary of MAP detection in the reproductive organs of bulls

References	Breed	Age (years)	Method of examination	Testicle	Epididymis	Seminal vesicle	Prostate gland	Bulbourethral gland
Larsen and Kopecky (1970)	n.s.	n.s.	ZN	-	n.e.	+	-	-
				-	n.e.	-	-	-
	n.s.	n.s.	ZN	-	n.e.	-	-	+
				-	n.e.	+	-	-
	n.s.	n.s.	ZN	-	n.e.	+	+	+
				-	n.e.	-	-	+
	n.s.	n.s.	ZN	-	n.e.	-	-	-
				-	n.e.	+	-	n.e.
	n.s.	n.s.	ZN	-	n.e.	-	+	n.e.
				-	n.e.	-	-	n.e.
	n.s.	n.s.	ZN	-	n.e.	+	+	n.e.
				-	n.e.	-	-	n.e.
Larsen et al. (1981)	Holstein-Friesian	4	CE	-	n.e.	+	+	n.e.
Ayele et al. (2004)	Holstein-Friesian	n.s.	CE	-	-	n.e.	n.e.	n.e.
	Piedmont	n.s.	CF	+	+	-	n.e.	-
	Hereford	n.s.	CE	+	+	+	n.e.	-
	Simmental	n.s.	CE	-	-	+	n.e.	-
Glawischnig et al. (2004)	Fleckvieh	6	CE	+	n.e.	n.e.	n.e.	n.e.
			PCR	+	n.e.	n.e.	n.e.	n.e.
Khol et al. (2010)	Piedmont	2,5	CE	-	+	+	n.e.	-

CE: Culture examination;  
 ZN: Ziehl-Neelsen staining;  
 n.s: Not specified;  
 n.e: Not examined

**Table 8:** Summary of MAP detection in semen.

References	Species	Methods of examination	Number of examined animals	Number of examined samples	Number of positive samples (%)
Larsen and Kopecky (1970)	Cattle	CE	1	1	1 (100.0)
Larsen et al. (1981)	Cattle	CE	1	31	8 (25.8)
Eppleston and Whittington (2001)	Sheep	CE	11	11	3 (27.3)
Ayele et al. (2004)	Cattle	CE	1	100	1 (1.0)
Buergelt et al. (2004)	Cattle	CE	1	2	1 (50.0)
Khol et al. (2010)	Cattle	CE	1	9	0 (0.0%)
		F57 qPCR		9	6 (66.7)
		IS900 qPCR		9	9 (100.0)
Sharifzadeh et al. (2010)	Cattle	nPCR	112	112	14 (12.5)
Münster et al. (2013b)	Cattle	CE	1	40	0 (0.0)
		snPCR		65	29 (44,6)
		qPCR		29*	20 (69.0)
Khamesipour et al. (2014)	Cattle	nPCR	83	83	8 (9,6)

CE: Culture examination;

ZN: Ziehl-Neelsen staining;

n.s: Not specified;

\*: Previously positive result by snPCR

*Transmission of MAP between species*

Another source of infection for domestic ruminants is wild animals, which are infected with MAP and spread the pathogen into shared habitats. By the use of different multitarget genotyping methods, such as short sequence repeat (SSR) analysis, mycobacterial interspersed repetitive-unit variable-number tandem-repeat (MIRU-VNTR) analysis, and restriction fragment length polymorphism analysis based on IS900 (IS900-RFLP), the transmission of MAP between wild-living red deer (*Cervus elaphus*) and farmed cattle herds, which shared the same habitats, has previously been demonstrated (Fritsch et al., 2012). In addition to several ruminant wildlife species, non-ruminates may also be infected with MAP. The best-studied non-ruminant MAP-reservoirs are the wild rabbits of Scotland (Beard et al., 2001b; Carta et al., 2013; Greig et al., 1999). The high prevalence of MAP in rabbits, especially those in East Scotland, is associated with difficulties in controlling paratuberculosis on cattle farms (Shaughnessy et al., 2013). MAP has also been detected in many other non-ruminant species (Anderson et al., 2007; Beard et al., 2001a; Corn et al., 2005; Gronesova et al., 2008; Münster et al., 2013a); however, the number of possible infected animal species remains unclear. In this study, the occurrence of MAP in rock hyraxes (**chapter III**) as well as in non-human primate species (**chapter II**) was examined. The presence of MAP in rock hyraxes as well as in the common marmoset was demonstrated here for the first time. These descriptions expand the list of animals that seems to be susceptible to an infection with MAP. However, the impact of such infected non-ruminant species on the spread of MAP to domestic ruminants or other animals is currently unknown. Infected non-ruminant animals often do not develop clinical symptoms, and pathological and histological changes typical for paratuberculosis have been reported in only a few cases, for example in rabbits, donkeys and non-human primates (Beard et al., 2001b; McClure et al., 1987; Stief et al., 2012; Zwick et al., 2002). As demonstrated in the present investigation (**chapter I**), a MAP infection in asymptomatic ruminants does not always trigger clinical and/or pathological changes. For asymptomatic, infected ruminants, it is known that MAP is excreted intermittently and in varying concentrations during the subclinical stage of the disease (Mitchell et al., 2015; Münster et al., 2013b). Therefore, it can be assumed that subclinically infected non-ruminants also excrete the pathogen intermittently and in varying concentrations. Results of other studies have substantiated this hypothesis, as it has previously been demonstrated that

subclinically infected non-ruminants can excrete pathogen concentrations of up to  $10^6$  MAP/g faeces in rabbits (Daniels et al., 2003) and in other animal species that were kept in a German zoological garden (Münster et al., 2013b). As mentioned above (general discussion; Section: Transmission of MAP via the faeces of calves), an estimated concentration of  $10^3$  MAP/animal is considered to elicit MAP infection in calves (Brotherston et al., 1961); however, the minimal infectious dose for non-ruminants is unknown. Due to the likely excretion of MAP in different concentrations, such asymptomatic non-ruminants may be sources for the unrecognized spread of MAP, on a species level and between species. For instance, in South Africa, paratuberculosis appears to be an emerging problem in sheep (Michel and Bastianello, 2000). In this area, rock hyraxes are widespread and are considered as an agricultural problem, because they compete with domestic livestock for available grazing space (Kolbe, 1967; Lensing, 1976). Due to shared habitats, it is possible that the transmission of MAP may occur between the rock hyraxes and the local livestock ruminants. Therefore, it is likely that the three wild-born rock hyraxes investigated here were naturally infected in South Africa, although it cannot completely be excluded that an infection with MAP occurred during the transport from South Africa to Germany and/or during quarantine. Further studies are required to ascertain the occurrence of MAP in the wildlife, to evaluate the potential function of the wildlife as a reservoir for MAP and the resulting impact on domestic livestock. To provide answers regarding possible routes of MAP transmission, the investigation of the MAP occurrence in local rock hyraxes, wild and domestic ruminants as well as non-ruminant species should include the analysis of MAP isolates by multitarget genotyping methods.

### ***Comparison of different antigen detection methods***

In three of the studies presented here (**chapters I, II and III**), MAP was more often detected by PCR than by culture examination. Analyses of the faeces, blood, semen, and tissue samples from the German Fleckvieh bull (**chapter I**) clearly illustrated this discrepancy. Many different factors can adversely affect the detectability of MAP. Primarily, the formation of cluster, inhomogeneous distribution and rather small numbers of MAP within the samples negatively affect the detectability of MAP by antigen detection methods. In addition to these reasons, detectability using culture approaches is negatively influenced by additional components. The required

decontamination, which suppresses the accompanying flora, has a negative impact on the viability of MAP (Bradner et al., 2013). Furthermore, the above-mentioned ability of MAP to merge into a VBNC stage may also explain the failure of MAP growth in this investigation. The recovery rate is negatively influenced by these factors, especially in the case of rather small numbers of MAP, which are known to be lower in animals without visible clinical signs than in clinically infected animals. Due to this, the sensitivity of MAP culture from the faeces of subclinically infected animals is considered to be 23-49%, whereas culture of MAP from animals with clinical signs of paratuberculosis is thought to reach a sensitivity of 98% (Nielsen and Toft, 2008). Discrepancy between the detection of MAP by PCR and culture has also been observed in many other studies (Kralik et al., 2014; Kralik et al., 2011; Münster et al., 2013b). For instance, one study investigated 1906 faecal samples of dairy cattle. In total, 875 (45.91%) and only 169 (8.87%) samples tested positive for the presence of MAP by IS900-based real-time PCR and culture, respectively (Kralik et al., 2011). Although the culture of MAP is currently considered the gold standard for diagnosis, it is necessary to remember that, especially in the case of subclinically infected animals, culture as well as PCR should be used to examine animal samples.

Methods conventionally used for the detection of MAP must be performed in well-equipped laboratories; these are ill-suited for use at the point of need. The often-used real-time PCR i) requires complex devices, ii) must be performed by highly qualified personnel and iii) the reagents must be stored at -20 °C. In contrast to real-time PCR, RPA assays are extremely fast (3-15 minutes) and all reagents are cold-chain independent. Several different RPA assays have been developed for the detection of pathogens, such as group B streptococci, *Brucella* or *Mycobacterium tuberculosis* (Boyle et al., 2014; Daher et al., 2014; Ren et al., 2016). The development and validation of an RPA assay for the detection of MAP DNA is mentioned in **chapter IV** of this thesis. The limit of detection was 16 DNA molecules using a molecular DNA standard, and approximately 500 fg of the entire MAP genome. In order to enable the use of RPA assays at the point of need, a mobile suitcase laboratory was developed in our division (Figure 12) (Abd El Wahed et al., 2015). The use of the RPA assay in combination with a fast DNA extraction protocol would allow the direct detection of MAP in the field. These technologies are particularly suitable for low-resource settings or direct, on-site testing by veterinarians. However, the development of an equivalent

fast DNA extraction protocol for the isolation of MAP is still needed. Furthermore, the combination of both methods (speed extraction and RPA) would also allow the directly analysis of environmental and faecal samples from different species in the field, as recommended in **chapter III** of this thesis.



**Figure 12:** The mobile suitcase laboratory. The mobile setup consists of two suitcases, one for nucleic acid extraction (left suitcase) and another for detection (right suitcase), which contains all reagents and equipment for the detection of the nucleic acids of infectious agents using RPA technology. All reagents used in the mobile laboratory are cold-chain independent and powered by solar battery, making it ideal for low-resource settings (Division of Microbiology and Animal Hygiene, Georg-August-University Göttingen)

### *Primates and Crohn's disease*

The aetiology of Crohn's disease is uncertain, and the involvement of MAP in this disease is controversial (Behr and Kapur, 2008; Feller et al., 2007). Meta-analyses have demonstrated that MAP is more often present in patients with Crohn's disease than in control patients (Abubakar et al., 2007; Feller et al., 2007); however, a causal link has not yet been demonstrated (Rudoler, 2004; Van Kruiningen, 1999). Non-human

primates are genetically and physiologically the most similar to humans. In this investigation (**chapter II**), MAP DNA was detected in two different non-human primate species, again confirming the susceptibility of non-human primates to MAP infection. The presence of MAP was only demonstrated in species that belong to the *Callitrichidae* family. Species belonging to this family are known to often develop chronic colitis when kept in captivity (Ludlage and Mansfield, 2003), which although occurring independently is largely related to the symptom-complex of Wasting Marmoset Syndrome (WMS). The symptoms of WMS in marmosets show clear parallels to the clinical picture of Crohn's disease in humans. Due to the clinical and pathological similarities, cottontop tamarins have previously been used as an animal model for scientific studies on ulcerative colitis and Crohn's disease (Clapp et al., 1991); unfortunately, the occurrence of MAP was not monitored in these studies. Hence, non-human primates, especially marmosets, should be analysed in detail for the presence of MAP and macroscopic and microscopic pathological changes, which may be due to MAP infection. Such examinations may promote further understanding of the mechanisms involved in inflammatory bowel diseases associated with MAP infection.

**CONCLUSION**

The following important conclusions were drawn in this investigation:

1. In a longitudinal study, an intermittent detection of MAP in faeces, semen and blood of a naturally infected, clinically asymptomatic German Fleckvieh bull was observed.
2. MAP was not detected on 65% of the sample days, and up to nine weeks in any matrices, demonstrating the difficulties in the confirmation of a MAP infection in asymptomatic, infected ruminants.
3. In asymptomatic, infected hosts, the presence of MAP was more frequently detected by PCR than by bacterial culture, independent of the investigated matrices.
4. Serologically negative shedders can be a source of infection for other animals.
5. A further source of infection is calves, which can excrete MAP in concentrations of up to  $6.25 \times 10^6$  genome equivalents/g faeces.
6. The first descriptions of the detection of MAP in rock hyraxes and a common marmoset were here given. This expands the list of animals that are known to be susceptible to an infection with MAP.
7. The manifestation of MAP in most body regions in infected but asymptomatic ruminates and non-ruminant hosts was demonstrated.
8. MAP may replicate within an asymptomatic, infected host without triggering typical pathohistological changes or clinical signs of paratuberculosis.
9. It can be assumed that asymptomatic, infected non-ruminants excrete MAP intermittently and in varying concentrations, as was here demonstrated in a bull.



10. The repeated detection of MAP in the bone marrow (Fleckvieh bull and common marmoset) may be an indication that, as described for *Mycobacterium tuberculosis*, viable but not culturable (VBNC) MAP cells persist in the mesenchyme cells of the bone marrow and play a key role in the establishment of a latent infection.
11. The detection of MAP in the male reproductive organs and semen of a German Fleckvieh bull supports the hypothesis that MAP may be transmitted via semen.
12. Worldwide, this is the second demonstration of the presence of MAP DNA within the reproductive organs of a naturally infected, non-ruminant species.
13. MAP seems to be a pathogen carried by free-living rock hyraxes in South Africa, and may be a potential source of infection for local livestock animals.
14. A sensitive (16 DNA molecules) and rapid (15 min) RPA assay was developed to allow the detection of MAP in poor settings or directly at the point of need.
15. Confirmation that non-human primates, which are genetically and physiologically similar to humans, are susceptible to MAP infection.

## **FUTURE PROSPECTS**

The further prospects for this work are:

1. Development of new strategies for the reliable diagnosis of subclinical paratuberculosis.
2. Investigation of the occurrence of MAP in the bone marrow of infected animals, in addition to the gastrointestinal tissues and lymph nodes, to substantiate the hypothesis that the bone marrow may provide a niche for the establishment of a latent MAP infection.
3. Development of a sensitive and specific method to detect viable MAP in semen, to monitor the occurrence of MAP in the semen of subclinical as well as clinically infected breeding bulls.
4. Test the influence of antibiotics in the various semen extenders, as well as the effect of the freezing process on the viability of MAP in semen.
5. Analysis of further animal species with a haemoendothelial placenta to investigate this route of transmission.
6. Monitoring of the occurrence of MAP in different wildlife species to identify further potential reservoirs.
7. Development of a speed extraction protocol of MAP in faeces, with the aim of implementing the RPA assay in this field.
8. Detailed analysis of further MAP-infected non-human primates to understand the mechanisms of inflammatory bowel disease.

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## **5 APPENDIX**

## LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers:

- I** FECHNER K., SCHÄFER, J., WIEGEL, C., LUDWIG, J., MÜNSTER, P., SHARIFI, A. R., WEMHEUER, W., CZERNY, C.-P. (2015): Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in a Subclinical Naturally Infected German Fleckvieh Bull. *Transboundary and emerging diseases*, 64(3), 916-928.
  
- II** FECHNER K., MÄTZ-RENSING, K., LAMPE, K., KAUP, F.-J., CZERNY, C.-P., SCHÄFER, J. (2017): Detection of *Mycobacterium avium* subsp. *paratuberculosis* by IS900 based PCR assays in non-human primates kept in Germany. *Journal of medical primatology*, DOI 10.1111/jmp.12270.
  
- III** FECHNER K., SCHÄFER, J., MÜNSTER, P., TERNES, K., DÖRING, S., VÖLKEL, I., KAUP, F.-J., CZERNY, C.-P. (2017): Detection of *Mycobacterium avium* subsp. *paratuberculosis* in rock hyraxes (*Procavia capensis*) imported from South Africa. *Journal of Zoo and Wildlife Medicine*, under review.
  
- IV** HANSEN, S., SCHÄFER, J., FECHNER, K., CZERNY, C.-P., ABD EL WAHED, A. (2016): Development of a recombinase polymerase amplification assay for rapid detection of the *Mycobacterium avium* subsp. *paratuberculosis*. *Plos one*, 11(12), e0168733.

## LIST OF PRESENTATIONS

**I 5. Arbeitstagung des nationalen Referenzlabors für Tuberkulose und des nationalen Referenzlabors für Paratuberkulose (29. – 30.01.2013 in Jena):**

FECHNER, K., VÖLKEL, I., MÜNSTER, P., V. BUCHHOLZ, A., WEMHEUER, W., CZERNY, C.-P.: “Vergleichende molekularbiologische Analysen von *Mycobacterium avium* ssp. *paratuberculosis*-Isolaten aus Hauswiederkäuern und Zootieren.”

**II 5. Arbeitstagung des nationalen Referenzlabors für Tuberkulose und des nationalen Referenzlabors für Paratuberkulose (29. – 30.01.2013 in Jena):**

VÖLKEL, I., SCHWARZ, D., SCHÜTTLER, J., WESTERNHORST, U., MÜNSTER, P., FECHNER, K., WEMHEUER, W., CZERNY, C.-P.: “Zytologische und hämatologische Parameter im Verlauf natürlicher Infektionen mit *Mycobacterium avium* ssp. *paratuberculosis* bei Besamungsbullen.”

**III DVG VET-Congress “Tagung der DVG-Fachgruppe Umwelt- und Tierhygiene” (06. – 10.09.2013 in Berlin):**

FECHNER, K., WEMHEUER, W., CZERNY, C.-P.: “Epidemiologische Untersuchungen zum Vorkommen von *Mycobacterium avium* ssp. *paratuberculosis* bei Hauswiederkäuern und Zootieren mittels IS900 basierten PCR-Verfahren.”

**IV 34. Tagung der DVG-Fachgruppe AVID “Veterinärmedizinische Infektionsdiagnostik; Schwerpunkt Bakteriologie” (09. – 11.09.2015 in Banz):**

FECHNER, K., SCHÄFER, J., BURCHARDT, S., SCHÖTTKER-WEGNER, H.-H., WERCKENTHIN, C., NORDHOFF, M., KLARMANN, D., WEMHEUER W., CZERNY, C.-P.: “Nachweis von *Mycobacterium avium* ssp. *paratuberculosis* in subklinisch infizierten Bullen”

**V National Symposium on Zoonose Research 2016** (13. – 14.10.2016 in Berlin):

FECHNER, K., MÄTZ-RENSING, K., LAMPE, K., KAUP, F.-J., CZERNY, C.-P., SCHÄFER, J.: “*Mycobacterium avium* ssp. *paratuberculosis* in non-human primates”



**LIST OF POSTERS**

- I 12<sup>th</sup> International Colloquium on Paratuberculosis** (22. – 26.06.2014, Parma, Italy): FECHNER, K., SCHÄFER, J., WEMHEUER, W., CZERNY, C. P.: “Detectability of *Mycobacterium avium* ssp. *paratuberculosis* in subclinically infected bulls.”
- II 34. Tagung der DVG-Fachgruppe AVID “Veterinärmedizinische Infektionsdiagnostik; Schwerpunkt Bakteriologie ”** (09. – 11.09.2015 in Banz):  
FECHNER, K., MÄTZ-RENSING, K., LAMPE, K., KAUP, F.-J., CZERNY, C.-P., SCHÄFER, J.: “Nachweis von *Mycobacterium avium* ssp. *paratuberculosis* in Gewebeproben von nicht humanen Primaten.”

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**DECLARATION**

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examination body either in its present or a similar form.

Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Göttingen, .....

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(Signature)

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(Name in block capitals)

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

Göttingen, .....

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(Signature)

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(Name in block capitals)