


Shedding of *Mycobacterium caprae* by wild red deer (*Cervus elaphus*) in the Bavarian alpine regions, Germany

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Abstract

The number of natural infections with *Mycobacterium caprae* in wildlife and in cattle in the Bavarian and Austrian alpine regions has increased over the last decade. Red deer (*Cervus elaphus*) have been recognized as maintenance reservoir; however, the transmission routes of *M. caprae* among and from naturally infected red deer are unknown. The unexpected high prevalence in some hot spot regions might suggest an effective indirect transmission of infection. Therefore, this study was undertaken to diagnose the occurrence of *M. caprae* in faeces and secretions of red deer in their natural habitat. A total of 2,806 red deer hunted in this region during 2014–2016 were included in this study. After pathological examination, organs (lymph nodes, lung, heart), excretions and secretions (faeces, urine, saliva and tonsil swabs) were further investigated by qPCR specific for *Mycobacterium tuberculosis* complex (MTC), *M. bovis* and *M. caprae*. Samples tested positive by qPCR were processed for culturing of mycobacteria. In total, 55 (2.0%) animals were confirmed positive for *M. caprae* by pathological examination, PCR and culturing of the affected organ material. With the exception of one sample, all of the secretion and excretion samples were negative for mycobacteria of the *Mycobacterium tuberculosis* complex (MTC). From one red deer, *M. caprae* could be isolated from the heart sac as well as from the faeces. Whole-genome sequencing confirmed that both strains were clonally related. This is the first confirmation that *M. caprae* can be shed with the faeces of a naturally infected red deer. However, further studies focusing on a higher number of infected animals, sample standardization and coordinated multiple sampling are necessary to improve the understanding of transmission routes under natural conditions.

KEYWORDS

bovine tuberculosis, faeces, *M. caprae*, MTC, qPCR, wildlife

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1 | INTRODUCTION

Tuberculosis is one of the most important infectious diseases of humans. Species belonging to the *Mycobacterium tuberculosis* complex (MTC) are *Mycobacterium tuberculosis*, *M. bovis*, *M. caprae*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. mungi*, *M. orygis*, *M. sunicattae*, *M. canettii* and *Dassie Bacillus* (Alexander et al., 2010; Huard et al., 2006). *M. tuberculosis* is the main cause of tuberculosis in humans worldwide. The causative agents of bovine tuberculosis (bTB), *M. bovis* and *M. caprae*, can infect a wide range of hosts including domestic animals such as cattle, sheep, goats, water buffaloes and wildlife such as red deer (Javed, Usman, Irfan, & Cagiola, 2006; Lopez-Olvera et al., 2013), roe deer, bison (Krzysiak et al., 2018), foxes (Zanella et al., 2008), wild boar (Matos et al., 2014) and badgers (Delahay et al., 2007; Tomlinson, Chambers, McDonald, & Delahay, 2015). *M. caprae* is classified as a unique species since 2003 (Aranaz, Cousins, Mateos, & Dominguez, 2003). Both bTB causing species pose the highest zoonotic potential among all members of MTC (Muller et al., 2013). Although great efforts have been undertaken to control bTB in industrial countries, it remains a serious infectious disease for animals that is difficult to eradicate. Humans can be infected by both species, either by direct contact with infected animals or by consumption of raw food of animal origin such as contaminated milk, meat and meat products (FLI, 2018; Torres-Gonzalez et al., 2013). The World Organization for Animal Health (OIE) has declared bTB in cattle as a notifiable disease (OIE, 2013) according to its potential to cause serious infections in humans and huge economic losses in animal production.

A reemergence of bTB in cattle in the Bavarian State (Germany) was first observed in 2007 (FLI, 2013, 2018). A high number of bTB outbreaks in this area were recorded in 2009, 2012 and 2013 with 18, 20 and 35 outbreaks, respectively, mostly in the Allgäu region (Swabia, Germany), where most of the cattle are pastured in the mountains during summer (FLI, 2018). The transmission of bTB between wildlife and pastured cattle has already been reported (de Lisle, Bengis, Schmitt, & O'Brien, 2002; O'Brien, Schmitt, Rudolph, & Nugent, 2011), and the potential transmission of MTC from wildlife to cattle in Europe has been confirmed to contribute significantly to this situation (Hardstaff, Marion, Hutchings, & White, 2014). Wildlife reservoir hosts of *M. bovis* are brushtail possums (*Trichosurus vulpecula*) in New Zealand, European badgers (*Meles meles*) in Great Britain and Ireland, African buffaloes (*Syncerus caffer*) in South Africa, wild boars (*Sus scrofa*) in the Iberian Peninsula and white-tailed deer (*Odocoileus virginianus*) in the United States (Palmer, 2013). Based on the results of previous studies (Domogalla et al., 2013; Fink et al., 2015; Schoepf et al., 2012), it is supposed that the new emerging cases of bTB in cattle in Tyrol, Austria, and in the southwestern Bavarian region of the Allgäu (Swabia) are likely to be linked to bTB-infected red deer.

During 2011–2013, a monitoring programme of the Coordination of European Research Area-Net (ERA-Net) on 'Tuberculosis in Alpine Wildlife' in four alpine nations (Austria, Switzerland, Germany, Italy) was carried out. One of the objectives of this programme was to

investigate the prevalence of bTB in red deer, and additionally in other wildlife species such as wild boar, chamois and roe deer. The results of different monitoring programmes (National surveys in Austria, Germany, European Research on Emerging and Major Infectious Diseases in Livestock Animals: EMIDA, 2013) showed that the prevalence of *M. caprae* in red deer in the Lechtal region (Austria) was $\geq 20\%$, and in the nearby area, southern Bavaria (Germany), it was 1%–4% (Büttner, Just, Neuendorf, Hörmansdorfer, & Zimmermann, 2013; Domogalla et al., 2013; EMIDA, 2013; Fink et al., 2015; Schoepf et al., 2012). Whole-genome sequencing of *M. caprae* isolates from infected red deer and cattle showed a high similarity, which led to the conclusion that a transmission between these animals can continuously occur (Domogalla et al., 2013). Although red deer and cattle live in close vicinity at alpine pastures, direct contact can be excluded. In the course of the monitoring programmes, tuberculosis-typical lesions in positive red deer were found mostly in single lymph nodes or were even non-visible lesions. A so-called 'active tuberculosis', which is the only stage of tuberculosis that is supposed to be infectious in humans, was rarely found in infected red deer. These findings are surprising since the high bTB prevalence in the hot spot regions suggests an efficient direct or indirect transmission within the deer population. On the contrary, transmission crossing the host species, for example from red deer to cattle or vice versa, is probably restricted to the indirect way. Besides contaminated pastures, salt lick stones, fodder or soil at feeding places may be sources of indirect infection (Santos, Almeida, Gortázar, & Correia-Neves, 2015a).

Therefore, the aim of this study was to investigate whether *M. caprae* is shed by secretions and/or excretions even if no active tuberculosis is diagnosed. Similar to a study investigating the excretion of *M. bovis* (Santos, Almeida, et al., 2015a), we analysed faeces, urine, saliva and tonsil swabs of a representative number of hunted red deer (*Cervus elaphus*) from regions in Germany with a high bTB prevalence using PCR based methods, culturing techniques and whole-genome sequencing.

2 | MATERIALS AND METHODS

2.1 | Samples

A total of 2,806 red deer (*Cervus elaphus*) hunted during 2014–2016 in two regions of the Free State of Bavaria (Germany) were investigated in this study. The first selected region with a high prevalence of bTB in red deer is located in Swabia and Southern Upper Bavaria (so-called Bavarian Alps/bTB hot spot region), and encompasses the counties Oberallgäu, Ostallgäu, Weilheim-Schongau, Garmisch-Partenkirchen, Bad Tölz-Wolfratshausen, Miesbach, Rosenheim, Traunstein and Berchtesgaden. For comparative investigations, the Bavarian Forest (counties: Freyung-Grafenau and Regen), located in Lower Bavaria, was chosen as low incidence or bTB-free reference region. Figure 1 provides a geographical overview of both regions.

After hunting, age and body condition of the animals were recorded. The following organs were removed for laboratory

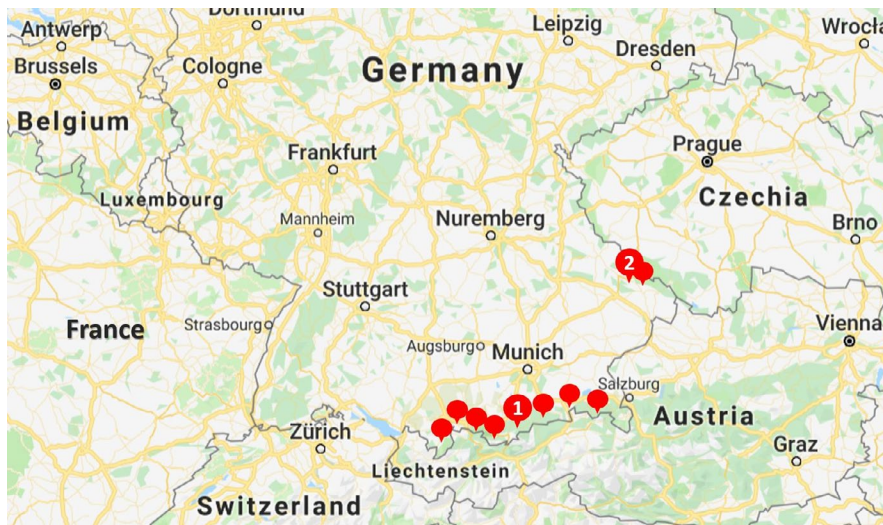


FIGURE 1 Hunting areas of red deer in this study: (1) Bavarian Alpine (bTB hot spot region), and (2) Bavarian Forest (low incidence region), in Bavaria, Germany [Colour figure can be viewed at wileyonlinelibrary.com]

investigation: whole head (alternatives: pharynx with retropharyngeal lymph nodes) and tonsils/tonsil swabs, lung and lung lymph nodes, heart, intestinal tract with mesenteric lymph nodes, saliva swabs and urinary bladder containing various volumes of urine. Faecal samples (approximately 100 g) were taken from the descending colon. All samples were kept at 4°C during transport to the Bavarian Health and Food Safety Authority (LGL, Bavaria, Germany) and investigated within 24 hr after hunting.

Table 1 shows the number of animals and derived samples (lymph nodes, faeces, urine, saliva swabs and tonsil swabs) investigated in this study. Organ samples (lymph nodes and other provided organs, e.g. head, pharynx, tonsils, lung, heart, intestine) were investigated by pathologists for bTB-typical lesions. The classification of animals as 'suspect' or 'non-suspect' for infection is based on the presence of bTB lesions in the investigated organs. Animals were classified as 'morphologically suspicious' if at least one organ showed characteristic lesions. If no suspicious lesion could be seen in any organ, the animal was classified as 'morphologically non-suspect'.

TABLE 1 Number of investigated animals and samples

Sample	Bavarian Alpine regions		Bavarian forest	
	Pos. ^a	Neg. ^b	Pos. ^a	Neg. ^b
Lymph nodes ^c	55	1,398	2	60
Faeces	19	931	-	79
Urine	15	526	-	75
Saliva swab	7	209	-	78
Tonsil swab	34	1,629	-	-
Total animal ^d	55	2,608	2	141

^abTB morphologically suspicious animals.

^bbTB morphologically non-suspect animals.

^cTracheobronchial and mesenteric lymph nodes.

^dTotal number of investigated animals differs from the number of investigated organs/samples since not all required samples from a single animal were delivered by hunters.

All samples (organs, excretions and secretions) from morphologically suspicious animals were subjected to DNA extraction and quantitative PCR (qPCR) amplification. Only samples positive for MTC by qPCR were further processed for culturing of the bTB agent in a biosafety level 3 laboratory (BSL 3) at the LGL. For animals classified as morphologically non-suspect for bTB infection, all excretion and secretion samples (faeces, urine, saliva and tonsil swabs, but not lymph nodes and organ samples) were further delivered to the Chair of Food Safety (LMU Munich, Germany), where DNA extraction and qPCR amplification for MTC were carried out.

Table 2 shows a list of bacterial species [*M. tuberculosis* complex ($n = 5$) and non-tuberculous mycobacteria ($n = 10$)], from which DNA was used for testing the specificity of the qPCR kit applied in this study. The reference species *Mycobacterium bovis* strain Bacille Calmette-Guérin-Pasteur (*M. bovis* BCG) was provided by the National Reference Center (NRC) for Mycobacteria, Research Center Borstel, Germany.

2.2 | DNA extraction

The DNA extraction of all excretion and secretion samples (faeces, urine, saliva and tonsil swabs) from morphologically non-suspect animals was performed at the Chair of Food Safety (LMU Munich). Urine samples were processed directly for DNA extraction. One gram of the faeces or pooled lymph nodes were suspended in a tube containing 9 ml NaCl and vortexed for 2 and 10 min, respectively. Swab samples were soaked in 1 ml NaCl for 10 min and then vortexed for 10 s. 140 µl of the samples/sample suspensions were processed for DNA extraction using the commercially available DNA extraction kit QIAmp[®] Viral RNA Mini Kit (Qiagen, Germany). The method of DNA extraction followed the instructions of the extraction kits, except excluding the step of DNA digestion. For samples from animals classified as morphologically bTB suspect (faeces, urine, saliva swab, tonsil swab, lymph nodes, lung and heart), the DNA extraction was performed at the LGL (Oberschleissheim) using the Gentra[®] Puregene[®] Yeast/Bact. Kit (Qiagen, Hilden, Germany).

TABLE 2 *Mycobacterium* species for the validation of the specificity of the qPCR kit

No.	Species	Source ^a
1	<i>M. bovis</i> BCG	NRC, LGL
2	<i>M. bovis</i>	LGL (V-08-402)
3	<i>M. bovis</i>	LGL (D 911/43990)
4	<i>M. africanum</i>	LGL (6,817)
5	<i>M. microti</i>	LGL (13-0086913-001-01)
6	<i>M. avium</i>	LGL (10-0165715-001-01)
7	<i>M. chelonae</i>	LGL (ERA 150)
8	<i>M. kansasii</i>	LGL (13-000617-003-01)
9	<i>M. intracellulare</i>	DSM 43223
10	<i>M. terrae</i>	DSM 43227
11	<i>M. scrofulaceum</i>	DSM 43992
12	<i>M. avium</i> subsp. <i>avium</i>	DSM 44156
13	<i>M. avium</i> subsp. <i>silvaticum</i>	DSM 44175
14	<i>M. avium</i> subsp. <i>asiaticum</i>	DSM 44297
15	<i>M. avium</i> subsp. <i>paratuberculosis</i>	DSM 44133

^aNRC: National Reference Center for Mycobacteria, Research Center Borstel, Germany; LGL: Bavarian Health and Food Safety Authority, Germany. The numbers shown in brackets are internal reference numbers; DSM: German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

All samples were extracted twice and processed independently for the MTC qPCR (see Section 2.3).

2.3 | qPCR protocol

Samples from animals classified as morphologically suspicious for bTB were processed for qPCR amplification with 4 qPCR systems prescribed by the Friedrich Loeffler Institute (FLI, 2015). These four systems are officially recognized in Germany in order to be applied for the diagnosis of MTC infections in cattle. The first and the second qPCRs aim to detect MTC (HELI + EK- and S1081 + EK-System; 91 and 85 bp, respectively); the third and the fourth qPCRs are applied to differentiate *M. bovis* and *M. caprae* (RD4 + EK- and MCAP + EK-System, respectively). Table 3 shows the sequences of primers and probes used for the detection of MTC, *M. bovis*, *M. caprae* and actin-DNA as an amplification control. Each 25 µl reaction of all 4 qPCR systems contained 0.8 pmol of each primer and 0.1 pmol probe (for MTC, *M. bovis* or *M. caprae*), 0.2 pmol of each primer and 0.1 pmol probe (for Actin-DNA), 12.5 µl of 2× QuantiTect Multiplex Master Mix, and 5 µl DNA template, filled up with RNA-free H₂O. The qPCR run was performed in a Stratagene® Mx3005P™ Real-Time Thermocycler (Agilent Technologies, Waldbronn, Germany). The 3-step qPCR of all 4 detection methods started with heating for initial denaturation at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for

60 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s. The results were categorized as positive, suspicious or negative.

The commercially available BactoReal® kit *Mycobacterium tuberculosis* complex (Ingentix, Austria) is approved to be used for the detection of MTC in various animal species in Austria. Handling of this kit is simple, and the results can be interpreted as positive (there is a PCR amplification signal) or negative (no PCR amplification signal). Thus, it was validated in comparison with the mandatory methods from the FLI (see Section 2.4). An exceptional permission from the FLI (as per § 11 Section 5 of the Animal Health Law; Bundesgesetzblatt, 2013) was issued that the qPCR kit mentioned above is allowed to be applied for the samples from morphologically non-suspicious animals in this study.

This qPCR kit with probes is specific for the internal transcribed spacer II (ITS2) region of MTC, and the fragment size of PCR products is 129 bp. Each reaction contained a 20 µl mixture that included 10 µl reaction mix (2×), 1 µl MTC assay mix (contains primers and probe for detection of MTC), 1 µl CR assay mix (contains primers and probe for the detection of the internal positive control, IPC), 3 µl H₂O and 5 µl DNA sample. The qPCR run was performed in the Bio-Rad CFX96 Touch™ Cycler (Bio-Rad Laboratories). A 2-step qPCR was applied, starting with incubation at 50°C for 2 min, followed by initial denaturation at 95°C for 5 min and 45 cycles in a series of denaturation at 95°C for 5 s; annealing and elongation were at 60°C for 60 s. PCR-positive samples tested with the BactoReal® kit were further differentiated to *M. bovis* or *M. caprae* using two further qPCR systems (RD4 + EK- and MCAP + EK-System, respectively), as described above.

2.4 | Limit of detection

An enumeration of *M. bovis* BCG was carried out. Mycobacteria usually stick together during growth, which leads to distorted results of both plate counting on solid medium and cell counting via microscope. To obtain single cells, *M. bovis* BCG was cultured in Middlebrook broth M7H9 (+ Supplement BBL™ Middlebrook ADC Enrichment, Becton Dickinson) and incubated aerobically at 37°C for about 3 weeks. The culture was shaken daily for 5 min. Before harvesting, the culture was allowed to rest overnight at 37°C. Then, only the supernatant of the culture was collected and filtered by using sterile gauze. The filtered supernatant was centrifuged at 500× g for 5 min, then transferred into a new tube and shaken for 2 min. The number of cells/ml was microscopically determined (400×) using a Thoma cell counting chamber. If bacterial cell clumps with more than 6 cells were still present, a centrifugation followed at up to 1,000× g for 10 min, and the enumeration of *M. bovis* BCG cells was re-assessed as described above.

After that, each type of sample suspension (lymph nodes, faeces, urine, saliva and tonsil swabs, see Section 2.2) from animals tested negative for MTC by qPCR was processed for artificial contamination with *M. bovis* BCG (10⁶–10¹ cells/ml sample suspension). DNA extraction and three qPCR systems were carried out as described in the Sections 2.2 and 2.3. The limit of detection of MTC in artificially

Specific qPCR for	Primer and probe	Sequence (direction 5'-3')
MTC (HELI + EK-System)	MTC-HELI-4F	TTG ATC AGG TCG ACG ATG TAG
	MTC-HELI-4R	TCA CCA CCG ACA AAG CGT C
	MTC-HELI-4FAM	FAM-TCA ACG ACC CCA ACG ACT GGT GC-BHQ1
MTC (S1081 + EK-System)	MTC-IS1081-5F	CTC TCG ACG TTC ATC GCC G
	MTC-IS1081-5Rn	TGG CGG TAG CCG TTG CGC
	MTC-IS1081-5FAM	FAM-ATT GGA CCG CTC ATC GCT GCG TTC-BHQ1
<i>M. bovis</i> (RD4 + EK-System)	MTC-RD4-6F	AAT GGT TTG GTC ATG ACG CCT TC
	MTC-RD4-6R	CGC CGT TGT AGG CCA CTC
	MTC-RD4-FAM	FAM-CAT ACA AGC CGT AGT CGT GCA GAA GC-BHQ1
<i>M. caprae</i> (MCAP + EK-System)	MTC-MCAP f	AGA CCG TGC GGA TCT TG
	MTC-MCAP r	CAT GGA GAT CAC CCG TGA
	MTC-CAP-FAM	FAM-TAT CGG GTA CAC AAA GAC GA-BHQ1
Actin-DNA (Amplification control)	ACT2-1030-F	AGC GCA AGT ACT CCG TGT G
	ACT-1135-R	CGG ACT CAT CGT ACT CCT GCT T
	ACT-1081-1105-YAK	YakimaYellow-TCG CTG TCC ACC TTC CAG CAG ATG T-BHQ1

TABLE 3 Primers and probes used for the detection of MTC and for the differentiation of *M. bovis* and *M. caprae* (FLI, 2015)

contaminated samples was determined with 3 qPCR systems. The first two qPCRs for MTC (HELI + EK- and S1081 + EK-System) are officially recognized in Germany. Thus, they were also included into the tests in order to compare the results with the third qPCR system (BactoReal[®] kit, Ingenetix, Austria).

2.5 | Isolation of *Mycobacterium* spp

Samples tested positive for MTC by qPCR were further processed for culturing as described in the handbook of diagnostics from the FLI (2015). One gram of each sample type (e.g. faeces, pooled lymph nodes) in 50-ml falcon tubes was added with 5 ml PBS and 5 ml N-acetyl-L-cysteine-NaOH solution (NALC-NaOH) to decontaminate the accompanying microorganisms. The sample suspension was shaken at 250 U/min for 20 min at room temperature. In order to neutralize NALC, the suspension was mixed with 10 ml Sorensen's buffer and centrifuged at 3,300× g for 20 min. The pellet was re-suspended in 1 ml Sorensen's buffer. Each 150 µl aliquot of the suspended pellets from NALC treated urine and lymph nodes was spread on Lowenstein-Jensen agar with glycerol and PACT (contains the antibiotics amphotericin, carbenicillin and malachite green), on Stonebrink agar with PACT, and added into Kirchner-Bouillon with PANTA (contains the antibiotics amphotericin, nalidixic acid and azlocillin). All 3 inoculated media (Artelt-Enclit GmbH & Co) are selective for mycobacteria and were incubated aerobically at 37°C. Since faeces contain a high level of intestinal bacteria, the samples were aliquoted into four subsamples in order to increase the chance to obtain MTC isolates. After treatment with NALC, 150 µl of the suspended pellets were added into a mycobacteria growth indicator tube (MGIT) containing 4 ml of modified

Middlebrook M7H9 broth base with OADC (Oleic acid, Albumin, Dextrose and Catalase) enrichment and PANTA antibiotic mixture. In total, 16 MGITs were used for an MTC isolation from one single faecal sample. If rapid microorganism growth (after 24–48 hr) was observed, NALC treatment was repeated, followed again by inoculation of MGI-tubes. All 16 tubes were incubated at 37°C in the MGIT incubator (Becton Dickinson). MGITs positive or suspicious for mycobacteria were further processed for MTC isolation using solid media as described above. The colony growth was investigated weekly until 8 weeks of incubation. Suspicious colonies were sub-cultured and subjected to DNA extraction and qPCR amplification specific for MTC, *M. bovis* and *M. caprae* (see Sections 2.2 and 2.3).

2.6 | Whole-genome sequencing

The DNA extracts of MTC isolates were further processed for whole-genome sequencing in order to search for intrahost heterogeneity in red deer.

Initially, 100 ng of each DNA was fragmented to an average length of 200 bp with M220 Ultrasonicator (Covaris). Sequencing libraries were then constructed from the fragmented DNA using single-stranded next-generation sequencing (NGS) Library Preparation Kit (Swift Bioscience) according to the manufacturer's instructions. Next-generation sequencing was performed in paired-end mode with the length of 100 bp in each direction with an Illumina HiSeq 1,500 (Illumina) sequencer. Sequenced reads were obtained in FASTQ format. Reads belonging to reverse direction were trimmed up to 15 nucleotides to remove bases additionally added by the library construction procedure. Reads were then

trimmed from both sides until the base quality was greater than Q20 and only those reads greater than 50 bp length after trimming were considered. Resultant reads were mapped against the assembly of *M. caprae* (NCBI reference sequence: NZ_CP016401.1) using BWA mem (Li & Durbin, 2009). Duplicated reads were removed using the Picard tool, and indels were realigned by GATK suite (McKenna et al., 2010). Finally, variants were called using VARSCAN (Koboldt et al., 2009) and manually corrected by inspecting visually in IGV (Thorvaldsdóttir, Robinson, & Mesirov, 2013) platform.

3 | RESULTS

Validation of the specificity and sensitivity of all 3 qPCR systems for MTC (HELI + EK-, S1081 + EK-System and BactoReal[®] kit) was carried out. All three systems could exclusively amplify DNA of all tested MTC species (*M. bovis*, *M. africanum* and *M. microti*), while there was no PCR amplification of DNA of non-tuberculous mycobacteria. Additionally, these 3 qPCR systems for MTC show a comparable limit of detection tested with *M. bovis* BCG, namely 1×10^2 cells/ml in suspensions of faeces, lymph nodes and swab samples, and 1×10^3 cells/ml in urine samples. Results of validation in this study support that all 3 qPCR systems for MTC show equal specificity and sensitivity. Because of easier handling and clear interpretation (positive or negative) with equivalent results compared to the methods described by the FLI (2015), the BactoReal[®] kit *Mycobacterium tuberculosis* complex (Ingenetix) was chosen for investigating all samples from morphologically non-suspicious animals (faeces, urine, saliva and tonsil swabs) in this study.

In total, 2,806 red deer were included in this study; 57 animals thereof ($n = 55$ from Bavarian alpine regions; $n = 2$ from the Bavarian Forest) showed similar pathological lesions typical for an infection with bTB in at least one investigated organ (e.g. abscesses or necrotic lesions in lung, tracheobronchial or mesenteric lymph nodes) and were classified as morphologically suspicious for bTB. The remaining 2,749 animals without typical signs or lesions were subsequently categorized as morphologically non-suspicious animals. Table 4 gives an overview of the qPCR results of all investigated samples (lymph nodes only from bTB suspicious animals, but all excretion and secretion samples). Since this study intended to investigate shedding of bTB via excretions and secretions, lymph nodes and other organs from morphologically non-suspicious animals were not further investigated by qPCR.

From 57 animals with bTB-typical lesions, 55 animals were tested positive for MTC by qPCR. That means, 2.1% of all investigated red deer ($n = 55/2,663$) from Bavarian alpine regions and 2.0% ($n = 55/2,806$) of all investigated red deer in both regions (Bavarian Alps and Bavarian Forest) were diagnosed as MTC-positive. In the Bavarian Forest region, the two animals with lesions in the lymph nodes suspicious for bTB infection were tested negative for MTC by qPCR. Of the 55 bTB-positive red deer, 19 (35%) were classified as old animals by the hunters.

Samples found to be positive for MTC by qPCR were mostly taken from organs with pathological lesions such as tracheobronchial and mesenteric lymph nodes, lung and heart (see Table 4). All samples positive for MTC were further differentiated as *M. caprae* using the qPCR procedure as described by the FLI (2015). After that, only samples tested positive for MTC by qPCR (e.g. lymph nodes and faeces) were processed for culturing, but not samples with negative MTC qPCR results.

An old female red deer (approximately 16 years) was PCR-positive for *M. caprae* in the organs (lung and tracheobronchial lymph nodes, heart sac, liver, spleen and mesenteric lymph nodes) and, additionally, in the faecal sample. *M. caprae* was isolated from the heart sac and from the faecal sample by cultivation. According to a high level of concomitant microorganisms in the faecal sample, the isolation of *M. caprae* was successful only after the third attempt. Subsequently, the strains from both samples were submitted for whole-genome sequence analysis.

About 10 million and 7.7 million reads were generated with the DNA extracted from the *M. caprae* isolates found in heart sac and faecal samples, respectively. The reads were mapped to the reference genome of *M. caprae* (99% mapping percentage), and a total of 398 variant positions were identified as a result of variant calling using Varscan. All variants that either occur in the repetitive genes (PE/PPE, IS and phage) of the *M. caprae* genome or found in both samples were discarded. Finally, the genomes of both samples differ in three variants only, that are shown in Figure 2. In the samples isolated from the heart sac, three single nucleotide polymorphisms (SNPs) were observed: position 337169:21%A-79%G; position 22217453:73%A-27%G; and position 3419776:62%A-38%G (the nucleotide found in the reference genome is underlined). The nucleotide sequences of the isolate obtained from faeces did not deviate from the reference genome at all three positions.

TABLE 4 Results of MTC qPCR from samples of all investigated red deer

bTB morphological evaluation	No. of animal	Number of samples positive by MTC qPCR				
		Lymph nodes	Faeces	Urine	Saliva swab	Tonsil swab
Suspicious	55 ^a	55	1	0	0	0
Suspicious	2 ^b	0	0	0	0	0
Non-suspicious	2,749 ^c	n.d.	0	0	0	0

Abbreviation: n.d.: PCR investigation was not done.

^aAnimals from Bavarian Alpine regions.

^bAnimals from Bavarian Forest.

^cTotal of bTB non-suspicious animals from both regions.

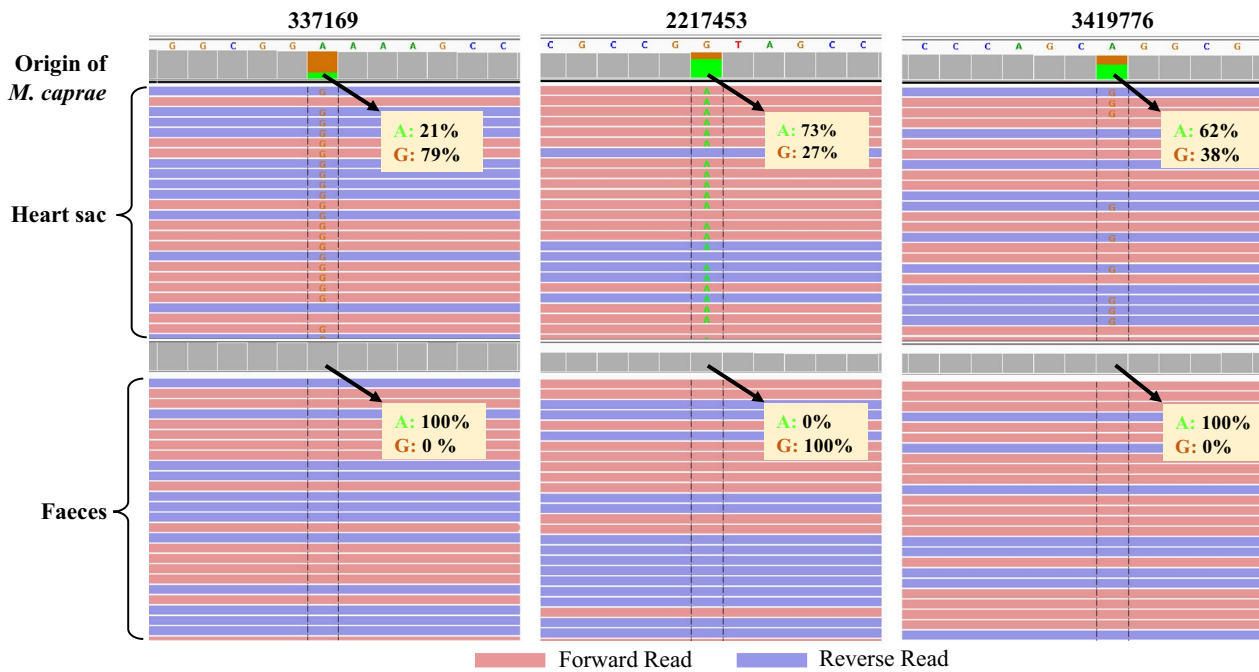


FIGURE 2 Single nucleotide polymorphisms (SNP) detected in the genome of *M. caprae* isolated from the heart sac and faecal sample of an infected red deer. Sequence reads obtained from mycobacterial genomic DNA isolated from heart sac and faecal samples of a single red deer were mapped against the *M. caprae* reference genome (NZ_CP016401.1). Arrows hint to allele frequencies inferred at the genomic position 337169, 2217453 and 3419776. Reads are coloured according to the matching strand (forward: red, reverse: blue) [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

In total, 2.0% ($n = 55/2,806$) of all investigated red deer were diagnosed as bTB-positive, determined by pathological examination and MTC qPCR. All bTB-positive animals identified in this study were hunted in the Bavarian alpine regions and were found to be infected with *M. caprae*. In 54 positive animals, the bTB-associated lesions were located only in lymph nodes. Only one positive red deer (female, about 16 years old) showed multiple tuberculous lesions in several organs including lung and heart. The cultural growth of *M. caprae* from the faecal sample and from the heart sac of this animal allowed whole-genome sequencing and a comparative analysis of two distantly located isolates within one host. Three heterozygous SNPs were found in the heart sac isolate. Thus, it can be concluded that the heart sac isolate contains a mixture of *M. caprae* descendants of *M. caprae* found in faeces. These differences were evaluated as marginal for MTC, since a few SNPs can occur especially in case of relapse ($\leq 6-10$ SNPs, Hatherell et al., 2016). The mutation rate of slowly growing MTC is assumed to be constant over time, approximately 1.09 SNPs per year (Guerra-Assunção et al., 2015; Hatherell et al., 2016). However, the chronic feature of bTB infection of this old red deer indicated that an infection in the juvenile age was likely. After infection, in this case, *M. caprae* was able to spread and infect many organs but slightly mutated during the long course of infection.

Culturing of *M. caprae* is challenging, even in morphologically positive organs. The isolation of MTC especially from faeces is even more problematic due to the high load of intestinal microorganisms, which

makes mycobacterial DNA detection more successful than culturing (Santos, Santos, et al., 2015b). This was also the case in this study: although the faecal sample was clearly positive for *M. caprae* by qPCR, the strain could only be isolated after the third round of isolation attempts, which is very time-consuming (up to 12 weeks). In order to avoid an unnecessary amount of work, samples (organs, lymph nodes, excretions and secretions, $n = 4,987$) negative for MTC by qPCR were not processed for culturing, regardless of whether they were from morphologically bTB suspicious or non-suspicious animals.

The detection of *M. bovis* in secretions and excretions from animals by using qPCR-based methods has been reported for, for example, faeces of naturally infected badgers in Great Britain (Sweeney et al., 2007) or red foxes in France (Michelet et al., 2018). In Portugal, faeces and urine samples of wild boars and red deer with suspicious lesions were also found positive for the MTC group by qPCR (Santos, Almeida, et al., 2015a). In this study, none of the animals was positive for *M. bovis*, since *M. caprae* is the dominant species of bTB established in the investigated areas (Fink et al., 2015; Müller et al., 2014; Schoepf et al., 2012). The occurrence of *M. caprae* infections is mostly reported in continental Europe, especially in Spain and in countries with alpine wildlife habitats (Domogalla et al., 2013). Besides red deer and cattle, *M. caprae* was detected in dromedary camels and bisons in a zoological garden in Slovenia (Pate et al., 2006), as well as in free-ranging European bisons in Poland (Krzysiak et al., 2018), wild boars in Hungary and Spain (Cšivincsik, Rónai, Nagy, Svéda, & Halász, 2016; García-Jiménez et al., 2013), and grey wolves in Poland (Orłowska et al., 2017). Recently, an *M. caprae*

infection outside Europe was reported by Yoshida et al. (2018) in a Borneo elephant in Japan.

Among wildlife species, red deer are often considered as spill-over hosts, which can occasionally spread MTC to other wildlife or domestic animals (Nugent, 2011; Wilson et al., 2009). However, in high-density populations, red deer can become maintenance hosts as demonstrated in the alpine regions of Austria and Germany (Fink et al., 2015; Wilson et al., 2009). In Swabia and Southern Upper Bavaria, the intensive investigation on bTB in cattle and wildlife such as red deer started after the noticeable increase of bTB outbreaks in cattle in 2009. A study of Domogalla et al. (2013) reveals that the genomic characteristics of *M. caprae* isolates from red deer were similar to isolates from cattle from the respective geographical regions in Southern Bavaria. Therefore, a transmission of *M. caprae* between cattle and red deer in this area is possible. However, there is scant information on epidemiological and transmission data of bTB in red deer, cattle and other animals. The prevalence of bTB in red deer (2.1%) in this study is higher than in cattle (0.19%) from the same area (Boenchendorf, 2016). According to Delahay et al. (2007), prevalence levels alone are unlikely to provide sufficient information to assess the potential risks of transmission to cattle. Further information such as amount of shed bacteria, amount of animal excretion, frequency of direct and indirect contact with cattle, population density and animal weight is required.

Considering the samples, especially excretions and secretions obtained from hunters in this study, standardized conditions in terms of quantity and quality could not be fulfilled satisfactorily. However, this is a general problem when hunting under alpine winter field conditions. Additionally, the DNA extraction methods could be optimized to detect MTC lower than 10^2 cells/ml sample suspension, since the amount of bTB shed in secretions and excretions can depend on the host species, health condition and on the status of manifestation such as active or non-active tuberculosis. A study of McCorry et al. (2005) on shedding of *M. bovis* in the nasal mucus of experimentally infected cattle showed that shedding of mycobacteria in secretions and excretions could be only in a low number and transient.

One old red deer investigated in this study showed generalized bTB lesions. *M. caprae* was detected and isolated from its faeces, pointing to a characterization as 'super shedder' (Santos, Almeida, et al., 2015a). Until now, there is no report about shedding of *M. caprae* via faeces in naturally infected red deer in Europe. Therefore, this is the first proof that *M. caprae* can be excreted via faeces under natural conditions. However, this kind of super shedder may be scarce, since old and at the same time severely infected animals often live solitary. Thus, the intra- and interhost transmission caused by this shedder may not be as effective as via young infected animals. In this study, at least 35% (19/55) of the bTB-positive red deer were classified as old animals and their social behaviour should be considered in terms of their bTB spreading potential to other animals.

Nevertheless, an indirect infection of wildlife and cattle on pastures may occur since soil can be contaminated with MTC, as recently reported from Spain (Barasona et al., 2017). Due to the high stability of mycobacteria in the environment, a contamination of grassland/

pastures in the Alps may contribute to indirect infection of wildlife and domestic animals being highly susceptible for *M. caprae* infections. However, the main transmission routes which could explain the high prevalence in some hot spot areas and the role of so-called 'super shedders' still remain unclear. To elucidate these questions, further studies including a higher number of naturally MTC infected red deer, sufficient quality and quantity of each sample, regular sampling intervals and enhanced analysis conditions (especially DNA extraction methods) are required to clarify whether animals infected with non-active tuberculosis actually shed the bacteria through secretions and excretions.

5 | CONCLUSIONS

This cross-sectional study was undertaken to investigate the possibility of MTC shedding via secretions and excretions of naturally bTB-infected red deer in Bavaria, Germany. In total, 2.0% (55/2,806) of the investigated red deer were positive for *M. caprae*. Only one bTB-positive animal excreted *M. caprae* via faeces. Two *M. caprae* stains, isolated from the faeces and from the heart sac of one old and generally affected animal, allowed comparative whole-genome sequencing and showed no major sequence mutations over a presumably long incubation period. Since shedding of bTB via secretions and excretions could be intermittent, a longitudinal study may be appropriate, however difficult to apply in free-ranging wildlife. Therefore, a high number of animals which are confirmed to be infected are required, and the isolation and DNA extraction methods should be optimized to detect a low number of MTC in secretions and excretions of infected red deer.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL STATEMENT

The study was conducted in compliance with ethical standards.

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