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Nontuberculous mycobacterial infections in animals and humans: pathogenesis, diagnosis, prevention, treatment, and epidemiology

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Published in
Frontiers in Veterinary Science
Frontiers in Public Health

ISSN 1664-8714
ISBN 978-2-8325-5831-7
DOI 10.3389/978-2-8325-5831-7

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Nontuberculous mycobacterial infections in animals and humans: pathogenesis, diagnosis, prevention, treatment, and epidemiology

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Citation

Marianelli, C., Ghielmetti, G., Pavlik, I., eds. (2024). *Nontuberculous mycobacterial infections in animals and humans: pathogenesis, diagnosis, prevention, treatment, and epidemiology*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-5831-7

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frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 10 December 2024
DOI 10.3389/fvets.2024.1532801

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 22 November 2024
ACCEPTED 28 November 2024
PUBLISHED 10 December 2024

CITATION
Marianelli C, Pavlik I and Ghielmetti G (2024) Editorial: Nontuberculous mycobacterial infections in animals and humans: pathogenesis, diagnosis, prevention, treatment, and epidemiology.
Front. Vet. Sci. 11:1532801.
doi: 10.3389/fvets.2024.1532801

Editorial: Nontuberculous mycobacterial infections in animals and humans: pathogenesis, diagnosis, prevention, treatment, and epidemiology

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KEYWORDS

nontuberculous mycobacteria, *Mycobacterium avium*, diagnostic tools, paratuberculosis, interference tuberculosis detection

Editorial on the Research Topic
Nontuberculous mycobacterial infections in animals and humans: pathogenesis, diagnosis, prevention, treatment, and epidemiology

Diseases caused by nontuberculous mycobacteria (NTM) represent a significant global public health concern. At present, NTM are defined as a group of environmental saprophytic organisms, comprising over 190 distinct species (1). Numerous NTM are opportunistic pathogens that can infect both animals and humans, resulting in a range of serious illnesses (2). Several studies have suggested that members of the *Mycobacterium avium* complex (MAC) are the most common NTM species identified in human infections and in particular in NTM lung disease (3). To date, MAC comprises 12 species, the most clinically relevant being *M. avium*, *M. intracellulare*, and *M. chimaera* (4). *Mycobacterium avium* is currently divided into four subspecies including *avium* (MAA), *silvaticum* (MAS), *hominissuis* (MAH), and *paratuberculosis* (MAP) based on virulence for animals and humans and other phenotypic peculiarities and specific genetic markers (4). MAH is the most clinically relevant to humans, often causing chronic pulmonary diseases and lymphadenitis in children and to pigs causing lymphadenitis in mesenteric and head (esp. submandibular) lymph nodes. MAP causes Johne's disease (paratuberculosis, PTB), a chronic granulomatous enteritis in ruminants. MAA and MAS have mostly been isolated from birds with tuberculosis-like disease.

The incidence of NTM diseases has been rising, and there is currently no established method of eliminating/eradicating the sources of infections from the environment and/or hosts (5–7). Furthermore, vaccines have yet to be developed. This Research Topic, entitled "*Nontuberculous mycobacterial infections in animals and humans: pathogenesis, diagnosis, prevention, treatment, and epidemiology*", presents a collection of 12 studies that explore various aspects of NTM infections. Several studies address the efficacy of diagnostic

tools for NTM diseases and possible interference with current *ante mortem* tuberculosis detection methods, others describe the occurrence of NTM in domestic and wild animals and the genetic diversity of NTM isolates from a wild population. Finally, a human case of NTM infection is also described.

The article by Zhang et al. reviews the applications and advancements in molecular diagnostics for identifying NTM species and subspecies. NTM infections are a growing public health concern, and accurate identification is crucial for effective treatment. Conventional methods, such as microbiological culture, are time-consuming and may not differentiate between closely related NTM subtypes. The manuscript discusses various molecular methods, emphasizing their key advantages over conventional microbiological methods, such as turnaround time, accuracy, and the ability to detect drug-resistance genes. Moreover, the article discusses the challenges in correlating *in vitro* drug susceptibility testing results with clinical outcomes. Finally, the potential of microfluidic technologies, artificial intelligence, and machine learning in enhancing the precision and efficiency of NTM identification and drug susceptibility testing, is also discussed. Stefanova et al. evaluate the efficacy of gross and microscopic investigations as diagnostic methods for MAP infection in non-vaccinated and anti-MAP vaccinated goats with subclinical infection. The article describes the prevalence of both gross PTB-compatible lesions and histopathological MAP-induced lesions in non-vaccinated goats. This finding suggests that anti-MAP vaccination may be beneficial in reducing PTB lesions and bacterial load in target organs. Furthermore, the concurrent utilization of both diagnostic tools enhances the detection of PTB lesions. Castro-Rodriguez et al. evaluate the diagnostic efficacy of two commercially available PCR-based kits for the identification and differentiation of *M. tuberculosis* complex (MTBC) and NTM human clinical isolates. The study reports a 100% sensitivity in the detection of MTBC for both kits, but a lower sensitivity for NTM identification. Gomez-Buendia et al. describe the NTM species that may act as a potential source of diagnostic interference to the intradermal tuberculin test (IDT), the most widely applied bovine tuberculosis test. The article characterizes MAH as the most abundant followed by MAA and *M. intracellulare* among MAC, and *M. nonchromogenicum* and *M. bourgelatii* as the predominant species among non-MAC members. The above four contributions highlight the limitations of the current diagnostic methods, including serological, cultural, histological, cell-mediated immunity, and molecular investigations. The combination of these methods may, however, increase the efficacy of the detection of NTM infections. Furthermore, vaccination may be a useful method of reducing the damage caused by some NTM diseases, such as PTB lesions.

Lienhard et al. examine the occurrence of NTM infection in wild animals from Switzerland, including red deer, roe deer, chamois, ibex, and badgers. The article emphasizes the opportunistic nature of numerous NTM species (particularly *M. vaccae* and MAH), which are unable to induce any macroscopic lesions in NTM-infected animals. Furthermore, the study describes the isolation of MAP from the head lymph nodes of two male red deer that exhibited no macroscopic lesions or clinical signs of disease. The study by Barandiaran et al. investigate the occurrence of NTM in four endangered Argentinian wildlife species, including giant anteater, peccary, tapir, and pampas deer. The giant anteater is identified as the species exhibiting the highest prevalence. The NTM identified include, among others, MAH and *M. intracellulare*, isolated from a range of mammalian hosts. The findings highlight the importance of NTM surveillance in conservation programs due to potential interference with tuberculosis diagnosis and public health implications. Komine et al. characterize nine *M. montefiorense* isolates from three salamander species. The strains' microbiological and genetic characteristics are analyzed, and the pathology of the infection in infected salamanders is described. The study contributes to understanding the genetic diversity and phenotypic characteristics of *M. montefiorense*, as well as the pathology of infection. In particular, phylogenetic analyses reveal that the isolates are genetically closely related, which could potentially indicate a common infection source. Turco et al. investigate the genetic diversity of *M. avium* field strains isolated from red deer in the *Stelvio* National Park in Italy. The main outcomes of this study are the genetic diversity and population structure of MAP isolates, which belong to a single major clade, indicating a clonal infection. The study also identifies two MAH isolates by investigating the same red deer population. The above four topic's articles report the occurrence of NTM and emphasize the genomic variation of NTM species in wildlife. The manuscripts contribute to our understanding of the genetic diversity and phylogenetic relationships of NTM species.

Ottardi et al. investigate the use of a commercial ELISA test in determining the seroprevalence of MAP infection in dairy herds and identifying risk factors associated with MAP spread. Because of the low seroprevalence within herds and low sensitivity of the ELISA test, the authors are unable to identify any reliable risk factors. Filippi et al. estimate the prevalence of PTB in red deer population over three culling seasons, and evaluate the relationship between the probability of being MAP-positive and individual and sampling-level variables. The large-scale serological survey by Di Marco Lo Presti et al. provides insights on MAP infection in sheep and goat herds in Sicily (Southern Italy). It reveals a high overall apparent prevalence of PTB at herd-level in sheep and goat farms, and reports animal-level prevalence data for both small ruminants. The study also indicates that the prevalence of PTB varies significantly between breeds of both sheep and goats.

While the majority of contributions to this topic focus on NTM infections in both domestic and wild animals, the study conducted by Sun et al.—which describes a rare human case of lymphadenitis caused by *M. chimaera*—underscores the fact that NTM infections represent a significant public health concern for humans as well.

Overall, these studies highlight the critical need for ongoing research and the development of comprehensive intervention strategies aimed at elucidating the mechanisms underlying NTM infections. These efforts are crucial for improving public health outcomes, enhancing veterinary management practices, and promoting effective wildlife conservation efforts, all within the context of a One Health Medicine and One Health approach.

Author contributions

CM: Writing – original draft, Writing – review & editing. IP: Writing – review & editing. GG: Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 26 October 2023
DOI 10.3389/fvets.2023.1248288

OPEN ACCESS

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RECEIVED 27 June 2023
ACCEPTED 22 September 2023
PUBLISHED 26 October 2023

CITATION
Komine T, Ihara H, Inohana M, Kwok JC, Shimizu A, Terasawa T, Miyazaki A, Srivorakul S, Iwao H, Harada S, Yoshida M, Hoshino Y, Kurata O, Fukano H and Wada S (2023) Non-tuberculous mycobacterial disease associated with *Mycobacterium montefiorense* in salamanders.
Front. Vet. Sci. 10:1248288.
doi: 10.3389/fvets.2023.1248288

Non-tuberculous mycobacterial disease associated with *Mycobacterium montefiorense* in salamanders

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Introduction: *Mycobacterium montefiorense* is one of the causes of non-tuberculous mycobacterial infections in moray eels and salamanders. Although *M. montefiorense* infection could be a threat to salamanders, little information is available regarding this pathogen and associated infection. This study aimed to provide fundamental information regarding *M. montefiorense* and its infection in salamanders.

Methods: Nine *M. montefiorense* strains isolated from three species of salamanders, namely, Japanese black salamander (*Hynobius nigrescens*), Hakuba salamander (*H. hidamontanus*), and Tohoku hynobiid salamander (*H. lichenatus*), between 2010 and 2018, were characterized based on phenotypic and genetic examination. We also pathologically observed salamanders infected with the *M. montefiorense* strains, including Hakuba salamanders and Tohoku hynobiid salamanders.

Results: The microbiological and chemical characteristics of the *M. montefiorense* salamander and an eel strain (reference strain) matched. Susceptibility testing for antimicrobials suggested that clarithromycin may be effective. Regarding disinfectants, phtharal, peracetic acid, glutaral, sodium hypochlorite, and benzalkonium chloride may be effective. Phylogenetic analyses revealed that the strains isolated from salamanders in 2014 and 2018 were genetically closely related, which could indicate an outbreak. The main gross findings in infected salamanders include skin ulcerative lesions or nodules in the enlarged liver. Microscopically, multifocal to coalescent granulomatous lesions composed of massive macrophages containing numerous acid-fast bacilli were prominently observed in the liver.

Conclusion: This study contributes to our understanding of the genetic diversity and phenotypic characteristics of *M. montefiorense*, as well as the pathology of the infection.

KEYWORDS

infection, pathology, whole-genome sequencing, pathogenesis, phylogeny, Mycobacterium montefiorense, salamander

1. Introduction

Non-tuberculous mycobacteria (NTM) are ubiquitous environmental organisms that can cause infection in humans and animals (1). In amphibians, mycobacteriosis has been associated with NTM species (*Mycobacterium marinum*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium xenopi*, and *Mycobacterium ulcerans* ecovar Liflandii), with infections primarily occurring in frogs in captivity (2–4). *Mycobacterium montefiorense*, a ubiquitous slow-growing NTM, belongs to the *Mycobacterium simiae* complex and is genetically closely related to *Mycobacterium triplex* (5–7). *M. montefiorense* was discovered in captive green moray eels (*Gymnothorax funebris*) and spotted moray eels (*G. moringa*) (8). *M. montefiorense*-associated mycobacteriosis has also been reported in Japanese black salamanders (*Hynobius nigrescens*) and Hakuba salamanders (*H. hidamontanus*) (9).

In Japan, out of the 90 evaluated amphibian species/subspecies, 67 species/subspecies are listed on the "Red List" by the Japanese Ministry of the Environment, and among them, 46 species/subspecies belong to the order *Caudata* (10). Research on the rearing and captive breeding methods and exhibition of these rare amphibians in zoos and aquariums is essential, not only for future conservation efforts but also for raising visitor awareness about the importance of conserving these species (11, 12). Infectious diseases, including *M. montefiorense* infection can damage captive amphibians (9, 13). However, research on *M. montefiorense* and its associated infection is limited.

Phenotypes (i.e., growth rate and temperature, pigment production, colony morphology, and biochemical characteristics) are important factors for mycobacterial species characterization and identification (14, 15). In addition, phenotypic data for antimicrobial susceptibility are crucial for effective antimicrobial treatment strategies (16, 17). Additionally, understanding bacterial susceptibility to disinfectants is critical for quarantine (18). However, *M. montefiorense* phenotypes are less studied. In this study, we evaluated phenotypic characteristics of *M. montefiorense* to provide fundamental phenotypic data.

Though phylogenetic taxonomy using 16S rRNA gene sequencing, which started to dominate the field of bacterial classification in the 1990s, is still a powerful tool, whole-genome sequencing has a higher resolution and can provide insights into exact species and subspecies present (19). In 2018 and 2022, draft genome sequences of *M. montefiorense* strains were reported (9, 20). However, genome-based phylogenetic analysis in detail has not been performed yet. In the present study, we performed phylogentic analyses by using whole-genome sequencing to confirm the phylogenetic relationship and position of *M. montefiorense* among closely related mycobacterial species.

Molecular epidemiological methods using whole genome sequencing data allow tracing transmission chains, identify super-spreaders, and predict undiagnosed transmission events, potentially leading to early treatment of infectious patients and prevention of pathogen spread (21–23). Furthermore, it could assess the effectiveness of intervention strategies for controlling infections (24). In this species, there is no insight into molecular epidemiology. Therefore, we conducted a whole-genome-based molecular epidemiological analysis in *M. montefiorense* strains isolated from 2010 to 2018 to uncover the infectious expansions in the salamander.

Clinical and pathological findings play a key role in the diagnosis (4, 25, 26). Only a few findings (granulomas in the liver and ulcers on the skin) in salamanders infected by *M. montefiorense* were reported (9). We pathologically evaluated infected salamanders with the mycobacterial species to provide more useful evidence/features for diagnosis.

Here, to provide fundamental information regarding the pathogen and pathology of the infection as described above, we evaluated the phenotypic and genetic characteristics of *M. montefiorense*, including strains ($n = 9$) isolated from salamanders, as well as pathology of the salamanders ($n = 9$) infected by *M. montefiorense*. This study will help to develop diagnostic and prevention protocols to control this infection.

2. Materials and methods

2.1. Bacterial strains

A total of 11 strains, including 9 *M. montefiorense* strains isolated from salamanders, 1 *M. montefiorense* ATCC BAA-256 (reference strain) from a moray eel, and 1 *M. triplex* JCM 14744 (reference strain), were used in this study (Table 1). The salamander strains were collected from the infectious cases in Niigata City Aquarium (Niigata, Japan) between 2010 and 2018, and the isolation and culturing method were as described in the previous papers (9, 20). Briefly, following American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2013 edition) (29), eight dead or euthanized salamanders in the aquarium were collected and routinely dissected. The liver tissues were sampled and frozen at −20°C until further examination. The tissues were thawed, homogenized, and decontaminated with 1 ml of Nacetyl-L-cysteine-sodium citrate-NaOH for no longer than 15 min. After neutralization with 6 mL of phosphate buffer (pH 6.8), the samples were centrifuged at 3,000 × *g* for 20 min; the obtained pellets were then inoculated in Middlebrook 7H10 agar supplemented with 10% BBL Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton, Dickinson and Company, USA) and in 2% Ogawa egg slants (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan). The media was incubated at 25°C for 2 months. Isolates obtained were identified as *M. montefiorense* based on the Runyon classification system (14), phylogenetic analysis of the 401-bp 65-kDa heat shock protein gene (*hsp*65) amplified with the Tb11/Tb12 primer set (Supplementary Figure S1) (30), and average nucleotide identity analysis using PyANI (31) in their whole-genome sequences.

Mycobacterium triplex, a *M. montefiorense* genetically close species, served as a control in several tests.

2.2. Microbiology and chemical biology

2.2.1. Preparation of bacterial strains

Strain stocks [stored at −80°C in 20% (v/v) glycerol] were inoculated onto Middlebrook 7H10 agar supplemented with 10% BD BBL™ Middlebrook Oleic Albumin Dextrose Catalase OADC Enrichment (Becton, Dickinson and Company, USA) and pre-cultured for 4 weeks at 25°C. Colonies were suspended in sterile phosphate-buffered saline (PBS) (−), and suspensions were adjusted to an optical

TABLE 1 Information on the strains used in this study.

Strain	Species	Year isolated	Isolate source	Geographic location	Reference
BS	*Mycobacterium montefiorense*	2010	*Hynobius nigrescens*	Japan	(9)
NJB14191	*M. montefiorense*	2014	*H. hidamontanus*	Japan	(27)
NJB14192	*M. montefiorense*	2014	*H. hidamontanus*	Japan	(27)
NJB14194	*M. montefiorense*	2014	*H. hidamontanus*	Japan	(27)
NJB14195	*M. montefiorense*	2014	*H. hidamontanus*	Japan	(27)
NJB14197	*M. montefiorense*	2014	*H. hidamontanus*	Japan	(27)
NJB18182	*M. montefiorense*	2018	*H. lichenatus*	Japan	(27)
NJB18183	*M. montefiorense*	2018	*H. lichenatus*	Japan	(27)
NJB18185	*M. montefiorense*	2018	*H. lichenatus*	Japan	(27)
ATCC BAA-256	*M. montefiorense*	NC	*Gymnothorax funebris*	USA	(5)
JCM 14744	*M. triplex*	NC	*Homo sapiens*	USA	(28)

NC, not clear.

density (OD_{530}) of 0.08–1.0. These suspensions were used for all experiments unless otherwise noted.

2.2.2. Growth rate and optimal growth temperature

Growth rate and optimal growth temperature were determined following the procedure described by the Japanese Society for Tuberculosis (2016) (32); however, Middlebrook 7H10 agar supplemented with 10% OADC enrichment was used. Suspensions (20 µl) were inoculated on Middlebrook 7H10 agar supplemented with 10% OADC enrichment. The media were incubated at 4, 25, and 37°C for 4 weeks and checked daily for the first week, then once weekly thereafter.

2.2.3. Pigmentation and chemical biology

The pigment production ability of all strains was tested on 2% Ogawa egg slants (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan) as described by Fukano et al. (33). Suspension was inoculated on two 2% Ogawa egg slants and cultured at 25°C for 2 months under dark conditions. One of the slants was irradiated with a 60 W fluorescent lamp at 30 cm for 1 h, and incubated at 25°C for 24 h under dark conditions again. After the procedure, the coloration of the bacterial colonies in the irradiated and non-irradiated slants was compared and pigment production ability was judged according to the Runyon classification system (14).

Urease production and catalase tests were conducted in eight strains (*M. montefiorense* BS, NJB14191, NJB14192, NJB14194, NJB14195, and NJB14197, ATCC BAA-256, and *M. triplex* JCM 14744), according to the procedure described by the Japanese Society for Tuberculosis (2016) (32). In the urease production test, a loopful of 2% Ogawa egg slant-grown colonies was resuspended in 2 mL of 1/100 M phosphate buffer (pH 6.8) supplemented with 3% urea and 0.001% neutral red and incubated at 25°C for 3 days. When the color of the solution changed to red, we interpreted it as positive. For the semiquantitative catalase test, 0.1 mL suspension was inoculated on 2% Ogawa egg slant in a tube of 18 mm × 180 mm and incubated for 3 weeks. After adding 0.5 mL of tween-peroxide solution (prepared by mixing equal volumes of 30% H_2O_2 and 10% Tween-80) to the media, the tubes were incubated at approximately 20°C for 5 min. Subsequently, the column of bubbles was measured. The test results were interpreted as follows: ≥45 mm high catalase reaction and < 45 mm low catalase reaction. In thermo-stable catalase test, a loopful of 2% Ogawa egg slant grown colonies was resuspended in 0.5 ml of 1/15 M phosphate buffer (pH 7.0) in test tubes (16 × 125 mm). Afterward, the tubes were incubated at 68°C for 20 min. After cooling, 0.5 ml of the tween-peroxide solution was added and the evolution of bubbles was observed. When the formation of bubbles was observed in 20 min, we interpreted it as a positive reaction.

2.3. Antimicrobial and disinfectant susceptibility

Amikacin and ciprofloxacin are used for treating bacterial infections in amphibians (34), and clarithromycin, rifampicin, streptomycin, kanamycin, and doxycycline for NTM diseases in humans (16, 35). Therefore, these seven antimicrobials were subjected to susceptibility tests. Clarithromycin (CAM, FUJIFILM Wako Pure Chemical Corporation, Japan), rifampicin (REF, Sigma-Aldrich, Merck KGaA, Germany), streptomycin (SM, Sigma-Aldrich), kanamycin (KM, Sigma-Aldrich), amikacin (AMK, Sigma-Aldrich), doxycycline (DOXY, Sigma-Aldrich), and ciprofloxacin (CPFX, MP Biomedicals, USA) susceptibilities were determined for the representative four strains (*M. montefiorense* BS, NJB14195, ATCC BAA-256, and *M. triplex* JCM 14744) using the standardized microdilution method for slowly growing mycobacteria as recommended in the Clinical and Laboratory Standards Institute guidelines (36). Minimal inhibitory concentrations (MICs) were determined after incubating inoculated microdilution plates at 25°C for 5 days. The breakpoints of CAM, REF, AMK, DOXY, and CPFX were judged according to the CLSI breakpoint criteria for slowly growing non-tuberculous mycobacteria (36), and those of SM and KM were judged according to the criteria for *M. tuberculosis* (37).

Disinfectant susceptibility was also determined for the four strains, following the methods previously described by Best et al. (38) and Hernández et al. (39). The disinfectants included 2% (W/V) glutaral

(STERIHYDE L, Maruishi Pharmaceutical. Co., Ltd., Japan), 0.55% (W/V) phtharal (DISOPA® Solution 0.55%, Johnson & Johnson KK, Japan), 0.3% (W/V) peracetic acid (ACECIDE, Saraya Co. Ltd., Japan), 75% (V/V) ethanol (FUJIFILM Wako Pure Chemical Corporation), sodium hypochlorite (2% of available chlorine concentration) (FUJIFILM Wako Pure Chemical Corporation), 0.5% (W/V) benzalkonium chloride (TEGO 51® Disinfectant Solution 30%, Alfresa Pharma Corporation, Japan), 0.5% (W/V) chlorhexidine gluconate (HIBITANE®, Sumitomo Pharma Co., Ltd., Japan), and 0.13% (W/V) didecyldimethylammonium chloride (Astop, Scientific Feed Laboratory Co., Ltd., Japan). The inoculum suspension (0.1 ml) was adjusted to an optical density (OD_{530}) of 0.08–1.0 and was treated with 0.9 ml of the disinfectants and added to 9.9 mL of a neutralizing agent or sterile dilute water. Neutralization or dilution was conducted at 1, 5, 10, 15, 30, and 60 min. Glutaral and phtharal were neutralized by 5% glycine and 0.5% sodium hydrogen sulfite, respectively. Peracetic acid and sodium hypochlorite were neutralized by 3% sodium thiosulfate. All neutralizing agents were purchased from FUJIFILM Wako Pure Chemical Corporation. The neutralized suspension (10 µL) was inoculated on Middlebrook 7H10 agar supplemented with 10% BBL Middlebrook OADC enrichment and incubated at 25°C for 4 weeks. Following incubation, when no colonies were macroscopically observed, we determined the adequate contact time required for disinfection. Disinfectant susceptibility was tested in duplicate, and the longer time was adopted as the adequate contact time.

2.4. Phylogenetic analyses

2.4.1. Core genome MLST analysis

Core genome multilocus sequence typing (cgMLST) analysis was performed in 29 *Mycobacterium* spp. strain set from the National Center for Biotechnology Information (NCBI) database, including nine salamander strains (9, 20) and the reference strain (*M. montefiorense* DSM 44602). The analysis was performed following the pipeline described by Atxaerandio-Landa et al. (40). Specifically, the assembled genome sequences from the NCBI database were assessed using CheckM taxonomy_wf v1.2.0 + galaxy0 (--rank genus *Mycobacterium*) (28). The sequences, assessed as >99% completeness and < 5% contamination, were reannotated using Prokka v1.14.6 (41), and general feature format (gff) files were produced. The gff files were analyzed using Roary v3.13.0 (42) for core genes. A maximum likelihood tree was constructed from the core gene alignment using the best-fitted nucleotide substitution model (the 29 *Mycobacterium* spp. strain set, GTR+ F + I + G4; the *M. montefiorense* strain set, GTR + F) in the IQtree web server[1] (43) and visualized with Interactive Tree of Life (iTOL).[2]

2.4.2. Linkage network analysis

Linkage network analysis (23, 44) using core single nucleotide polymorphisms (SNPs) in the ten *M. montefiorense* strains was performed, based on clustering in the maximum likelihood phylogeny. *M. montefiorense* ATCC BAA-256 was used as an outgroup strain. Briefly, short-read sequencing data from nine strains of *M. montefiorense*, including strains from salamanders, were obtained from the NCBI database (Supplementary Table S1). The quality of raw reads was assessed with FastQC v0.11.9 (45). Core SNPs were called from the fastp-trimmed data with Snippy v4.6.0 + galaxy0 using the draft genome of *M. montefiorense* BS (5,744,567 bp, BFCH00000000) as a reference, and core SNP alignment, including the reference, was then generated using Snippy-core v4.6.0 + galaxy0.[3] The reference was selected based on the quality of sequences (Supplementary Table S1). From the core SNP alignment, pairwise SNP distances were calculated. Subsequently, a median joining network (46) was generated based on the SNPs of the core alignment in pop art v1.7 (47). Furthermore, to improve the resolution of the analysis, a median-joining analysis in the salamander strains within the same cluster (Figure 1A) was also conducted. Default parameters were used for all software unless otherwise noted.

2.5. Pathology

Salamanders, including Hakuba salamanders ($n = 5$) and Tohoku hynobiid salamanders ($n = 4$), that had been reared and exhibited in the Niigata City Aquarium and diagnosed as *M. montefiorense* infection, were subjected to histopathological examination. Of them, the salamander strains NJB14191, NJB14192, NJB14194, NJB14195, NJB14197, NJB18182, NJB18183, and NJB18185 were isolated (see Supplementary Table S2) (20). The examined salamanders in this study were hatched from wild-collected eggs and were reared in the aquarium (Supplementary Appendix S1; Supplementary Figure S2).

The salamanders were routinely dissected, and their external and internal gross features (reproductive organ, and/or alimentary tract) and fixed in a 10% phosphate-buffered formalin solution. The formalin-fixed tissues were processed routinely to prepare paraffin sections for histopathology. The sections were stained with hematoxylin and eosin (H&E) and Ziehl–Neelsen (ZN) stains.

3. Results

3.1. Microbiology and chemical biology

In growth rate and temperature, pigmentation, and chemical biology tests, the *M. montefiorense* strains from salamanders showed characteristics similar to those of *M. montefiorense* ATCC BAA-256 (Table 2).

3.2. Antimicrobial and disinfectant susceptibility

MICs of seven antimicrobials are summarized in Table 3, and all strains of *M. montefiorense* were susceptible to CAM according to the CLSI breakpoint criteria (36). Against the other six antimicrobials (REF, SM, KM, AMK, DOX, CPFX), *M. montefiorense* strains showed intermediate resistance, according to the criteria (36, 37).

1 https://iqtree.cibiv.univie.ac.at

2 https://itol.embl.de/

3 https://github.com/tseemann/snippy

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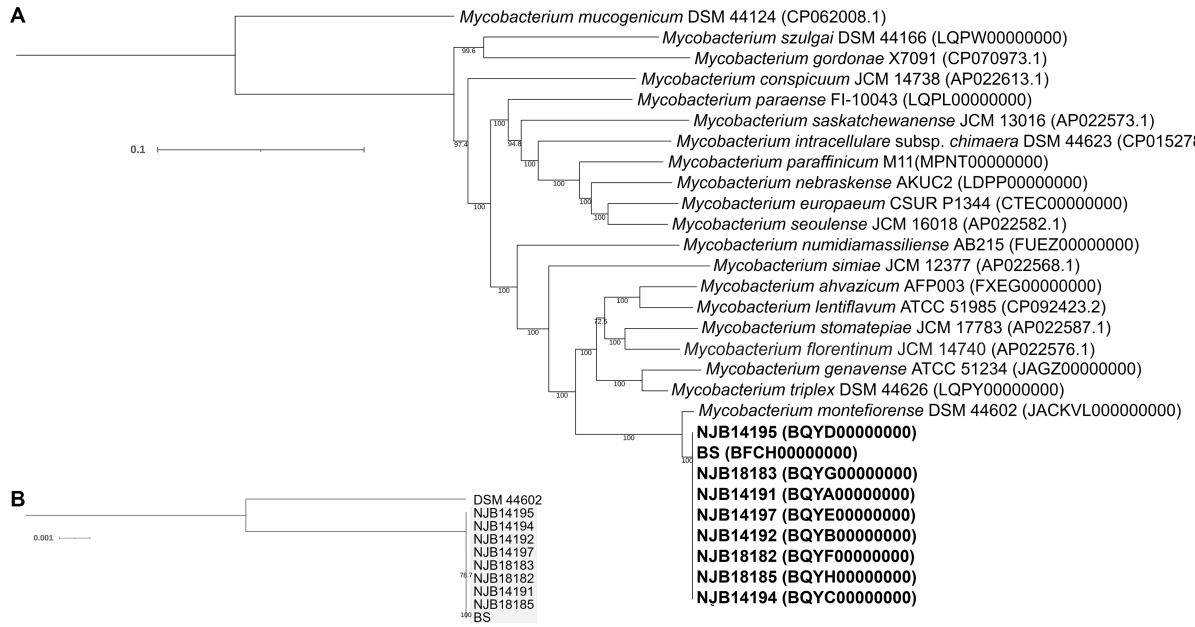

FIGURE 1
Phylogenetic analysis of *Mycobacterium montefiorense*. **(A,B)** Maximum likelihood phylogenies in the 29 *Mycobacterium* spp. strains and the *M. montefiorense* strains. The tree is midpoint rooted. Scale bars indicate nucleotide diversity.

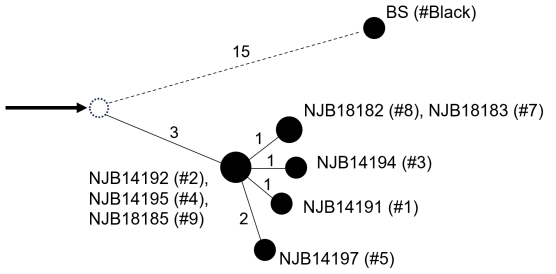

FIGURE 2
Median joining network in the 10 *Mycobacterium montefiorense* strains. Single nucleotide polymorphisms (SNPs) between strains are shown by numbers. Arrow denotes an outgroup strain. A dashed circle denotes a hypothetical common ancestor. # Denotes the ID numbers of the salamanders.

Adequate contact times of eight disinfectants are listed in Table 4. Four *M. montefiorense* strains were disinfected with phtharal and peracetic acid for ≤5 min, and with glutaral, sodium hypochlorite, and benzalkonium chloride for ≤60 min. Ethanol, didecyldimethylammonium chloride, and chlorhexidine gluconate did not disinfect certain strains with ≤60 min exposure.

3.3. Phylogenetic analyses

The cgMLST phylogeny based on 92 core genes (83,316 bp) showed that *M. montefiorense* was most closely related to *M. triplex* (Figure 1A). The cgMLST analysis based on the 4,630 core genes (4,669,478 bp) in the *M. montefiorense* strains indicated that the strains from isolated salamanders were classified into the same cluster, separated from the fish strain (Figure 1B). For pairwise SNP distances in the *M. montefiorense* strains, a total of 88,507 SNP loci were detected among 5,114,752 bp of the alignment of the *M. montefiorense* strain sequences. In the salamander strains, ≤15 SNPs were present, and the intragroup variation of the strains isolated in 2014 and in 2018 was 0–3 SNPs (see Supplementary Table S3). In the median joining network, BS and salamander strains (NJB14192, NJB14195, and NJB18185) show an accumulation of unique 15 SNPs and 3 SNPs, respectively, from a hypothetical common ancestor (Figure 2). Furthermore, from the three salamander strains, the other strains show an accumulation of unique 1–2 SNPs.

In addition, for pairwise SNP distances in a broader range of core regions using the salamander strain set, a total of 23 SNP loci were detected in 5,463,489 bp of the alignment of the salamander strain sequences (Supplementary Table S4; Supplementary Figure S1). Strain BS varied from the other salamander strains by 18–20 SNPs, and the other strains had 0–3 SNPs between each other.

3.4. Clinical signs and gross features

The dead salamanders showed only loss of energy and anorexia on the day before death. Six diseased Hakuba and Tohoku hynobiid salamanders showed ulcerative skin lesions on the neck, extremities, body side, and/or tail (Table 5). The livers of six salamanders were enlarged. In the three Hakuba salamanders, the livers were associated with multiple or coalescent gray-to-black nodules. In the livers of Tohoku hynobiid salamanders, multiple coalescent tan to white nodules were observed. Splenomegaly with multifocal-coalescent white nodules was also seen in five salamanders.

TABLE 2 Microbiological and chemical characteristics.

	Date isolated	Source	Growth rate	Growth temperature			Colony	Pigmentation	Catalase test	Catalase test (68°C)	Urease production test
				4°C	25°C	37°C					
Mm BS	2010	Salamander	SG	–	+	+	S	NP	L	–	–
Mm NJB14191	2014	Salamander	SG	+	+	+	S	NP	L	–	–
Mm NJB14192	2014	Salamander	SG	+	+	+	S	NP	L	–	–
Mm NJB14194	2014	Salamander	SG	+	+	+	S	NP	L	–	–
Mm NJB14195	2014	Salamander	SG	+	+	+	S	NP	L	–	–
Mm NJB14197	2014	Salamander	SG	+	+	–	S	NP	L	–	–
Mm NJB18182	2018	Salamander	SG	+	+	+	S	NP	ND	ND	ND
Mm NJB18183	2018	Salamander	SG	+	+	+	S	NP	ND	ND	ND
Mm NJB18185	2018	Salamander	SG	+	+	+	S	NP	ND	ND	ND
Mm ATCC BAA-256	NC	Fish	SG	+	+	+	S	NP	L	–	–
Mt JCM 14744	NC	Human	SG	+	+	+	S	NP	L	+	+

Mm, *Mycobacterium montefiorense*; Mt, M. triplex; SG, slow growing; S, smooth; L, low catalase reaction; NP, non-photochromogenic; ND, not done; NC, not clear.

TABLE 3 Minimum inhibitory concentrations (MICs) of antimicrobials against mycobacterial species.

Strain	Antimicrobial*						
	CAM	REF	SM	KM	AMK	DOXY	CPFX
Mm BS	4 (S)	>32 (R)	128 (R)	8 (R)	>64 (R)	>128 (R)	4 (R)
Mm NJB14195	2 (S)	32 (R)	64 (R)	32 (R)	64 (R)	>128 (R)	4 (R)
Mm ATCC BAA-256	2 (S)	>32 (R)	8 (R)	8 (R)	32 (I)	>128 (R)	4 (R)
Mt JCM 14744	16 (I)	>32 (R)	32 (R)	>128 (R)	>64 (R)	>128 (R)	>32 (R)

*Minimum inhibitory concentrations (μg/ml). Mm, *Mycobacterium montefiorense*; Mt, M. triplex; CAM, clarithromycin; REF, rifampicin; SM, streptomycin; KM, kanamycin; AMK, amikacin; DOXY, doxycycline; CPFX, ciprofloxacin S, susceptible; I, intermediate; R, resistant: The breakpoints of CAM, REF, AMK, DOXY, and CPFX were judged according to the CLSI breakpoint criteria of slowly growing non-tuberculous mycobacteria (45), and those of SM and KM were according to the criteria in *M. tuberculosis* (46).

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TABLE 4 Adequate contact time of disinfectants for mycobacterial species.

	Glutaral	Phtharal	Peracetic acid	Ethanol	Sodium hypochlorite	Benzalkonium chloride	Chlorhexidine gluconate	Didecyl dimethyl ammonium chloride
Mm BS	15 < t ≤ 30	<1	1 < t ≤ 5	>60	30 < t ≤ 60	30 < t ≤ 60	>60	>60
Mm NJB14195	10 < t ≤ 15	<1	<1	>60	1 < t ≤ 5	10 < t ≤ 15	>60	>60
Mm ATCC BAA-256	30 < t ≤ 60	<1	<1	>60	15 < t ≤ 30	15 < t ≤ 30	>60	>60
Mt JCM 14744	>60	1 < t ≤ 5	1 < t ≤ 5	>60	15 < t ≤ 30	>60	>60	>60

Mm, *Mycobacterium montefiorense*; Mt, M. triplex; t, minimum time (min) required to disinfect (no colony grew after treatment).

3.5. Histopathology

The most prominent histopathological features included multifocal to coalescent granulomatous lesions observed in the livers of eight salamanders with numerous acid-fast bacilli (Figure 2). Granulomas exist in the hepatic parenchyma and hematopoietic tissue of the liver, and most of the hepatic parenchyma was affected by the multifocal to coalescent lesions replacing normal parenchyma. These granulomas were characterized by aggregates of round to polygonal macrophages with abundant pale staining or occasional foamy cytoplasm and an eccentric round-to-ovoid nucleus, mixed with few lymphocytes and granulocytes. Numerous 1.5–3 × 0.3–0.5-μm sized acid-fast bacilli were often present within the cytoplasm of macrophages. In three salamanders, mild to severe multifocal granulomas with numerous acid-fast bacilli were observed in the systemic organs (i.e., skin, spleen, liver, kidney, reproductive organ, and alimentary tract) (Table 5). Granulomas in the splenic parenchyma were also seen. In the skin lesion, the granulomas were observed from the dermis to subcutaneous tissue (Figure 3). In a salamander that did not have the ulcer lesions, some macrophages with acid-fast bacilli were sporadically present in the dermis. In the kidney, the granulomas were located in the interstitium, and extracellular acid-fast bacterial colonies and bacteria within tubular epithelial cells were observed. In addition, macrophages containing acid-fast bacilli were also detected in the lamina propria to serosa in the alimentary tract and the lumen, lamina propria, and serosa of the oviduct. In a Tohoku hynobiid salamander, occasional multinucleated giant cells (Langhans type) were also observed in the liver. In one case, a granuloma was associated with a necrotic center containing numerous acid-fast bacilli.

4. Discussion

Mycobacterium montefiorense-caused mycobacteriosis in moray eels was first reported in 2001, where the pathogen was characterized based on molecular biological analyses for several genes, mycolic acids, and phenotypic data such as growth and biochemical composition (5, 8). Although Fukano et al. and Komine et al. reported the draft genome of the *M. montefiorense* strains from salamander species (9, 13), the pathology- and phenotype-related information was limited for the case. This is the first report on the pathology of mycobacteriosis associated with *M. montefiorense* in salamanders, along with detailed phenotypic characteristics such as drug susceptibility and genetic characteristics of this pathogen. Microbiological and chemical characteristic evaluations were similar between the isolated strains from salamanders and the type strain isolated from a moray eel, although the salamander strains varied from the eel strain by at least 88,000 SNPs. Therefore, these phenotypic characteristics could be stable in *M. montefiorense*, and are useful to identify this mycobacterium species (Figure 4).

As there are no effective treatments for mycobacterial infections in amphibians and there is a risk of zoonosis, euthanasia is recommended (3). However, *in vitro* susceptibility testing in this study has suggested that CAM could be effective against mycobacteriosis associated with *M. montefiorense* and other species of the *M. simiae* complex, despite the high levels of observed natural resistance (48). Similar to other veterinary diseases with zoonotic potential, attempting treatment with caution, as long as personnel administering treatment apply correct personal protection, is plausible. However, to our knowledge, no cases of CAM use have been reported in amphibians. Further study into the clinical effectiveness and side effects of CAM treatment would therefore be required.

NTM are ubiquitous agents that are isolated from environmental sources, and *M. montefiorense* has been isolated from water, aquatic plants, and sediment from ponds (6). Reducing pathogen numbers in patient environments using disinfection could prevent infectious diseases (1, 49). However, *Mycobacterium* spp. is known to pose resistance to disinfectants, and an intermediate- or high-level disinfectant is generally required (18, 50, 51). The disinfectant susceptibility testing in this study suggested the effectiveness of three high-level disinfectants: phtharal and peracetic acid in 5 min and glutaral in 60 min, as well as sodium hypochlorite, an intermediate-level disinfectant in 60 min and benzalkonium chloride, a low-level disinfectant, in 60 min. These time durations are similar to those recommended for disinfection of *M. tuberculosis* (18). It should be noted that *M. montefiorense* is highly resistant to ethanol, which is often used to disinfect equipment; therefore, ethanol is not considered appropriate for the disinfection of *M. montefiorense*.

In this study, the cgMLST phylogeny based on the 92 core genes confirmed that *M. montefiorense* is genetically closely related to *M. triplex*, supporting the insights of the previous papers (5–7, 20). Furthermore, the phylogeny of the 4,630 core genes suggested that the salamander strains form a cluster that is separated from a fish strain (ATCC BAA-256).

SNP analysis using whole-genome sequences has currently the highest level of resolution and is used for tracking transmission (23,

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TABLE 5 Histopathology data.

No.	species	Date collected	Dead/ euthanized	Gross lesions	Histopathology					
					L	Sp	AT	Sk	K	RO
1	*Hynobius hidamontanus*	10/20/2014	Euthanized	Skin ulcer in the dorsal side of the tail and left lateral neck. Hepatomegaly with multifocal-coalescent gray-black nodules. Splenomegaly with multifocal-coalescent white nodules.	+	NE	–	+	NE	–
2	*H. hidamontanus*	10/20/2014	Euthanized	Skin ulcer in the arms and right leg. Hepatomegaly with multifocal-coalescent gray-black nodules. Splenomegaly with multifocal-coalescent white nodules	+	NE	–	+	NE	–
3	*H. hidamontanus*	10/20/2014	Euthanized	Skin ulcer in the right forearm. Hepatomegaly with multifocal-coalescent gray-black nodules. Multifocal white nodules in the spleen	+	NE	–	–	NE	–
4	*H. hidamontanus*	10/20/2014	Euthanized	Skin ulcer in the ventral side of the abdomen. Hepatomegaly with multifocal-coalescent gray-black nodules.	+	NE	–	+	NE	–
5	*H. hidamontanus*	10/20/2014	Euthanized	–	+	NE	–	NE	NE	–
6	*Hynobius lichenatus*	3/28/2018	Dead	Hepatomegaly with multifocal-coalescent white nodules. Splenomegaly with multifocal-coalescent white nodules	+	+	+	+	+	+
7	*H. lichenatus*	8/26/2018	Dead	Skin ulcer in the dorsal side of the tail. Multifocal-coalescent gray-black nodules in the liver. Splenomegaly with multifocal white nodules.	+	+	+	+	+	+
8	*H. lichenatus*	8/28/2018	Euthanized	–	–	–	–	–	–	–
9	*H. lichenatus*	8/28/2018	Dead	Skin ulcer in the right foot. Hepatomegaly with multifocal-coalescent white nodules. Splenomegaly with multifocal- coalescent white nodules.	+	+	+	+	+	+

NE, not examined; NA, not applicable; L, liver; Sp, spleen; AT, alimentary tract; Sk, skin; K, kidney; RO, Reproductive organ. + Acid-fast bacilli were observed.

44, 52, 53). In the linkage network analysis of the *M. montefiorense* strains including ATCC BAA-256 as an outgroup, intragroup variation of strains within the cluster in the cgMLST analysis was ≤15 SNPs. The intragroup variation of the strains isolated in 2014 and in 2018 was 0–3 SNPs. There are no studies on the definition of a genetic relatedness cut-off (SNP values) and mutation rate in DNA sequences per genome per year in *M. montefiorense*. However, a cut-off value of 12 SNPs is often used and it has been suggested that the rate of genetic changes is approximately 0.5 SNPs per genome per year in latent, active, as well as re-activated diseases (21, 54, 55). A study of *M. chimaera* revealed a maximum of 38 SNPs among strains related to an outbreak (56). Specifically, 20–30 SNPs are defined as a cut-off

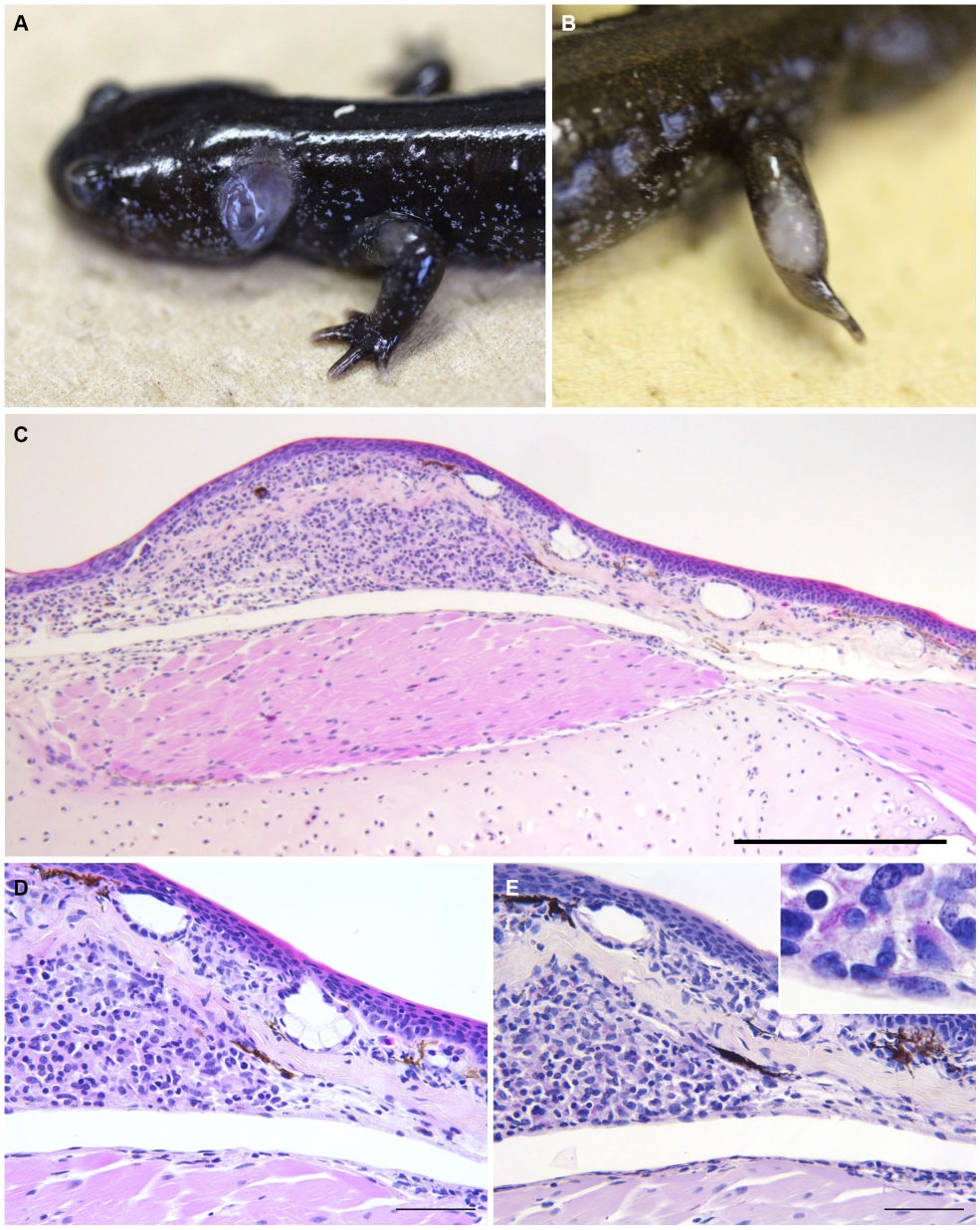

FIGURE 3
Gross and histopathological skin lesion in infected salamanders. **(A,B)** Skin legion in Hakuba salamanders (**A**, #1; **B**, #3). **(C,D)** The skin lesion of Hakuba salamander #4. Granulomas composed of aggregates of round to polygonal macrophages with abundant pale staining or occasional foamy cytoplasm and an eccentric round to ovoid nucleus, mixed with few lymphocytes and granulocytes were observed from the dermis to subcutaneous tissue (**C**, H&E stain, scale bar = 500 μm; **D**, H&E stain, scale bar = 100 μm). **(E)** Macrophages contain numerous acid-fast bacilli (ZN stain, scale bar = 100 μm).

value for transmission in *M. abscessus*, a rapidly growing mycobacterium (57–59). In addition, given that ≤3 SNPs were present in the strains isolated from salamanders collected on the same day in 2014 that had been reared in the same place and 0–3 SNPs were found between the strains in 2014 and the strains in 2018; therefore, these strains could be regarded as "possibly related." This molecular epidemiological result supported the epidemiological suggestion that there is a possible epidemiological link between the salamanders in 2014 and 2018 (see Supplementary Appendix S1; Supplementary Figure S2). We therefore estimated the situation in 2014 and 2018 could be an outbreak. Moreover, we speculated that the infection could have spread from individuals #2, #4, and #9 to individuals #1, #3, #5, #7, and #8 in chronological order, although the transmission route is unclear. In the aquarium, infected salamanders were identified and removed, and disinfection of the equipment suspected to be contaminated by the pathogen with boiling water was conducted as an intervention to control this infection from 2012. Since 2019, no infection in salamanders has occurred. This indicates that basic infection control strategies, such as the detection and removal of infected animals and disinfection of rearing facilities and the environment, were potentially effective against this infection. However, because of the difficulty in detecting infected individuals, where specific clinical signs are lacking, it could take a long period (several years) for the infection situation to resolve. Molecular

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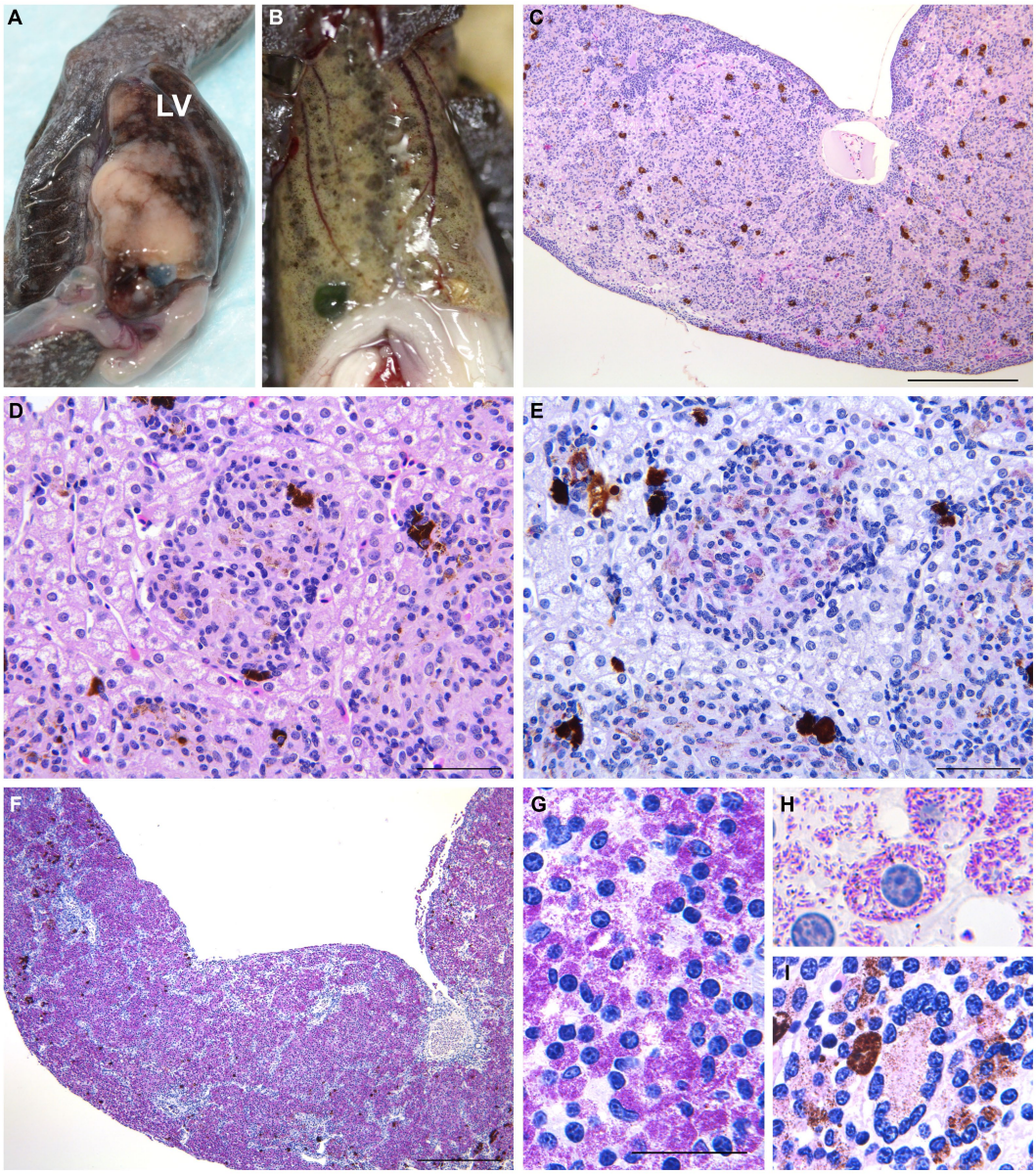

FIGURE 4
Gross and histopathological legion in the liver in infected salamanders. **(A)** Variably sized granulomatous nodules within the liver of a Tohoku hynobiid salamander #9. LV, liver. **(B)** Gray to black nodules in the liver of a Tohoku hynobiid salamander #3. **(C,D)** Multifocal nodular aggregates of histiocytes, resulting in hepatocyte compression and atrophy, were observed in a Hakuba salamander (#4). These granulomas were composed of aggregates of round to polygonal macrophages with abundant pale staining or occasional foamy cytoplasm and an eccentric round to ovoid nucleus, mixed with few lymphocytes and granulocytes (**C**, H&E stain, scale bar = 500 μm; **D**, H&E stain, scale bar = 100 μm). **(E)** Numerous acid-fast bacilli observed within the macrophages (ZN stain, scale bar = 100 μm). **(F)** More than 90% of the parenchyma is affected by coalescent granulomatous lesions replacing normal hepatic parenchyma in salamander #9. **(G,H)** Acid-fast bacilli 1.5–3 × 0.3–0.5 μm in size (**G**, ZN stain, scale bar = 50 μm; **H**, ZN stain, ×1,000). **(I)** Multinucleated giant cell observed in the liver of Tohoku hynobiid salamander #6 (ZN stain, ×400).

epidemiological approaches with whole genome sequencing are now contributing to the control of infectious diseases in humans, enabling the identification of transmission routes and sources and the evaluation of interventions (21–24, 60–62). This study suggests that the use of molecular epidemiological approaches could contribute to controlling infections, in zoos and aquariums as well.

In amphibian mycobacteriosis, nodules are generally formed on the skin and subcutaneous lymph sacs, with abscess formation and ulceration. In abdominal organs, white to yellow nodules are locally or multifocally observed, especially in the liver, spleen, and kidney (4, 63–66). Histologically, multifocal to coalescent granulomas were observed in the salamander organs affected by mycobacteria. Granulomas are commonly composed of large macrophages (epithelial cells), mixed with few neutrophils, eosinophils, and lymphocytes. In some cases, a few multinucleated giant cells were present. Serous granuloma formation can be seen in chronic lesions or cases caused by mycolactone-producing mycobacteria (i.e., *M. ulcerans* ecovar Liflandii). Chronic granulomas are surrounded by fibrous capsules, and acid-fast bacilli are often found within macrophages (4, 63–66). Although the granulomas of the salamanders in this study had no

fibrous capsules, the gross and pathological features were generally consistent with the findings commonly seen in mycobacteriosis of amphibians, as described above. Furthermore, our histopathological findings were similar to those of *M. montefiorense* infections in eels (8). These findings suggest that it is difficult to determine *M. montefiorense* as the causative agent of mycobacteriosis from pathological findings alone. Additionally, the detection of pathogenic genes from lesions and/or cultures of pathogens is necessary for diagnosis.

This study has a few limitations. First, this was a single-site study with a limited sample size. Second, the *in vivo* efficiency of antimicrobials in salamanders is uncertain, as only *in vitro* susceptibility testing for antimicrobials was conducted and the interpretation of the results (susceptible, intermediate, resistant) was based on the CLSI breakpoint criteria for humans. Third, the cut-off value for transmission and genome mutation rate per year in *M. montefiorense* remains uncertain. In this study, based on that among the strains isolated from tank-shared salamanders in 2014 and that of the other slowly growing mycobacteria, we judged the number of SNPs to be relevant at ≤3 (21, 54–56). Fourth, intrusion and transmission routes (salamander to salamander/environmental sources to salamander) were unclear. To address these limitations, the accumulation of infectious cases and further studies on the *in vivo* effectiveness of antimicrobials and molecular epidemiology in *M. montefiorense* are required. Nevertheless, we believe that this study provides novel and essential data on *M. montefiorense* and the associated infection.

In conclusion, this study characterized the mycobacterial strains isolated from salamanders based on phenotypic and genetic examination and the pathology of mycobacteriosis infection by *M. montefiorense* in salamanders. The study provides valuable information to diagnose and handle *M. montefiorense* infection as follows:

1. Microbiological and chemical characteristic evaluation findings were similar between the isolated strains (from salamanders) and the type strain (isolated from a moray eel).
2. Susceptibility testing for antimicrobials suggested that CAM-based treatment may be effective.
3. Disinfectant susceptibility testing suggested that phtharal, peracetic acid, glutaral, sodium hypochlorite, and benzalkonium chloride may be effective.
4. Phylogenetic analyses revealed that *M. montefiorense* strains isolated from the salamanders between 2014 and 2018 were genetically closely related, which could indicate an outbreak.
5. The main findings include skin ulcerative lesions or nodules in the enlarged liver in infected salamanders.
6. Multifocal to coalescent granulomatous lesions composed of massive macrophages containing numerous acid-fast bacilli in the liver were prominently observed.

This study will help clarify the genetic diversity and phenotypic characteristics of *M. montefiorense*, as well as the pathology of the associated infection.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was approved by National Institute of Infectious Diseases (NIID) Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HF and SW conceptualized the manuscript. TK wrote the original draft preparation. TK, HIh, MI, JK, AS, HF, AM, TT, and SS performed the pathological examination, microbiological and molecular analyses. HIw and SH managed the animals and collected the samples and epidemiological data. MY and YH revised the study. All authors read and approved the submitted version.

Funding

This study was supported in part by grants from the Japan Agency for Medical Research and Development/Japan International Cooperation Agency (AMED) to YH (JP20fk0108064, JP20fk0108075, JP21fk0108093, JP21fk0108129, JP21fk0108608, JP21jm0510004, JP21wm0125007, JP21wm0225004, JP21wm0325003, JP22gm1610003, JP22wm0225022, JP22wm0325054), to HF (JP22wm0325054).

Acknowledgments

We would like to thank Editage (www.editage.com) for English language editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at:https://www.frontiersin.org/articles/10.3389/fvets.2023.1248288/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 04 January 2024
DOI 10.3389/fvets.2023.1321106

OPEN ACCESS

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RECEIVED 13 October 2023
ACCEPTED 24 November 2023
PUBLISHED 04 January 2024

CITATION
Lienhard J, Friedel U, Paganini C, Hilbe M, Scherrer S and Schmitt S (2024) Isolation of *Mycobacterium avium* ssp. *paratuberculosis* and other non-tuberculous mycobacteria from head lymph nodes of wild ruminants and badgers in Switzerland.
Front. Vet. Sci. 10:1321106.
doi: 10.3389/fvets.2023.1321106

Isolation of *Mycobacterium avium* ssp. *paratuberculosis* and other non-tuberculous mycobacteria from head lymph nodes of wild ruminants and badgers in Switzerland

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Introduction: The family *Mycobacteriaceae* contains over 188 species, most of which are saprophytic non-tuberculous mycobacteria (NTM). In wildlife, a variety of different NTM can be found, with different reports about their pathogenic potential. A pathogenic member of NTM is *Mycobacterium avium* ssp. *paratuberculosis* (MAP), which can infect farmed and wild ruminants. It causes paratuberculosis which is an economically important chronic disease. Infected farm animals are considered to be the source of infection in wild animals. Wildlife, on the other hand, is thought to be a reservoir for certain members of the *Mycobacterium tuberculosis* complex (MTBC), such as *M. caprae*, which causes tuberculosis in cattle and red deer.

Methods: Switzerland implemented a surveillance program for tuberculosis in wild animals in 2014. Here, we describe the results from the mycobacterial culture of lymph node samples collected from red deer, roe deer, chamois, ibex, and badgers collected within this surveillance program from 2020 to 2022. Overall, samples from 548 animals were checked macroscopically for tuberculosis-like lesions.

Results: In total, 88 animals (16.1%), which either had lesions in their lymph nodes or were male and aged older than 5 years, were investigated using mycobacterial culture. In total, 25 animals (28.4%) were positive for NTM, while no MTBC was detected. The most often identified NTM was *M. vaccae*, followed by *M. avium*. Most animals positive for NTM did not show any macroscopic lesions. Furthermore, MAP was isolated from the head lymph nodes of two male red deer. Neither of the two MAP-positive animals had any macroscopic lesions in their head lymph nodes or any other signs of disease.

Discussion: The shooting sites of the two MAP-positive animals were located in Alpine pastures used for grazing of cattle during summer, which confirms that species transmission can occur when contaminated pastures are used by different species. In agreement with other studies, the occurrence of MAP in red deer was quite low. However, so far, MAP was mostly isolated from feces and intestinal lymph nodes of wild animals. This is the first detection of MAP in the head lymph nodes of red deer in Switzerland.

KEYWORDS

***Mycobacterium avium* ssp. *paratuberculosis*, non-tuberculous mycobacteria, red deer, wild ruminants, badger, retropharyngeal lymph node, mandibular lymph node, mycobacterial culture**

1 Introduction

The family *Mycobacteriaceae* contains over 188 species, most of which are saprophytes found in the environment. Saprophytic mycobacteria belong to the group of non-tuberculous mycobacteria (NTM), and many of them are opportunistic pathogens (1). In wild animals, a variety of different NTM can be found. Frequently found species include *Mycobacterium* (*M.*) *non-chromogenicum*, *M. peregrinum,* and *M. scrofulaceum* (2–4). In Switzerland, only data regarding wild boars are available, in which *M. avium* ssp. *hominissuis* is found most frequently, followed by *M. nonchromogenicum* (5). While some infected animals appear healthy without any visible lesions, others have granulomatous lesions in their lymph nodes (2, 3). However, there are also obligate pathogenic NTM, such as *Mycobacterium avium* ssp. *paratuberculosis* (MAP), which causes the chronic disease paratuberculosis in ruminants. In cattle, the disease causes diarrhea, weight loss, and reduced milk yield, which leads to economic losses (1). Furthermore, cattle are thought to be the source of infections of MAP in red deer (6). Infected red deer can develop typical clinical signs of paratuberculosis; however, they can also remain asymptomatic (7). The mean prevalence of MAP in wildlife in different countries is low at only 2.4%, however, there is quite a big range, i.e., from 0 to 100% (8). In Swiss red deer, the prevalence of MAP seems to be low as well (9).

Another important group of mycobacteria is the *Mycobacterium tuberculosis* complex (MTBC), which causes tuberculosis in humans and animals (1). Bovine tuberculosis is caused by *M. bovis* and *M. caprae*. However, other species such as *M. tuberculosis* and *M. microti* can also infect ruminants (10, 11). Due to its zoonotic potential and economic losses, many countries have introduced eradication programs for bovine tuberculosis (10). While Switzerland is officially free of bovine tuberculosis, the risk of recurrence of the disease remains as it was the case in two different outbreaks in 2013. One of these cases was caused by *M. bovis*, which seems to have persisted within a herd of animals for 15 years (12, 13). The other outbreak was caused by the *M. caprae* Lechtal genotype, which is endemic in Austrian red deer and dairy cattle (12). While *M. caprae* was not found in red deer in Switzerland before, there is a hot-spot area of red deer infected with *M. caprae* in Vorarlberg, Austria, close to the Swiss border (14, 15). As members of the MTBC can survive in the environment for a long period of time and indirect oral transmission is possible (16), it seems likely that the Swiss cattle got infected while summering on Austrian pastures contaminated with *M. caprae* (12, 14).

Red deer are considered reservoir hosts for tuberculosis and pose a source of infection for cattle (17). As a result of the outbreak of *M. caprae* in Swiss cattle in 2013, a surveillance program for tuberculosis in wild animals in Switzerland was implemented in 2014 by the Swiss Federal Food Safety and Veterinary Office and the Swiss cantons Grisons and St. Gallen and the Principality of Liechtenstein (12). As various wild animals, such as badgers, wild boar, red fox, and cervids, can be infected with bovine tuberculosis (18, 19), the program includes a risk-based approach, for which dead or diseased wildlife (red deer, chamois, ibex, roe deer, wild boar, foxes, and badger) are examined throughout the year. Furthermore, the program includes a random sampling approach, which examines red deer shot during hunting season. The sampling region covers the Swiss cantons of Grisons and St. Gallen and the Principality of Liechtenstein. Retropharyngeal and mesenteric lymph nodes are the primary sites of infection for tuberculosis, and lesions occur most often in these two lymph nodes (14). Hence, retropharyngeal and mandibular lymph nodes from all animals were collected and for the risk-based group, other lymph nodes, such as thoracic or mesenteric lymph nodes, were collected as well.

This study analyzes the data from the surveillance program from 2020 to 2022. While no mycobacteria from the MTBC were detected, various NTM, including MAP, were isolated. To the best of our knowledge, this is the first detection of MAP in the head lymph nodes of red deer in Switzerland. Furthermore, while NTM were detected in red deer in Switzerland before (15), this is the first study that identifies different NTM species found in red deer and wild animals other than wild boars in Switzerland.

2 Materials and methods

2.1 Sample collection

The samples used in this study were collected for a surveillance program for tuberculosis in wild animals in Switzerland, which was implemented in June 2014. In this study, only samples collected from 2020 to 2022 are included. This is due to the fact that starting from 2020, lymph nodes from male animals older than 5 years were directly analyzed using mycobacterial culture, irrespective of their macroscopic appearance. This change was implemented to increase the sensitivity of the surveillance, as older male animals are at a higher risk of infection with tuberculosis (20). In the majority of cases, lymph nodes were collected by veterinarians in the slaughterhouse, otherwise by hunters in the field.

Overall, for the risk-based approach, lymph node and organ samples were collected from 35 wild animals, as shown in Table 1. The random sampling group contained lymph node samples from 513 red deer, as shown in Table 2. Retropharyngeal and mandibular lymph nodes from all animals were collected. Additionally, for the risk-based group, organs with lesions and their associated lymph nodes, such as thoracic or mesenteric lymph nodes, were collected as well. However, in a few cases of the risk-based approach, no head lymph nodes were collected; thus, in these cases, only the organs with lesions and their associated lymph nodes were examined.

2.2 Macroscopic evaluation of the lymph nodes

Upon arrival in the laboratory, the tissue surrounding the lymph nodes was removed. The lymph nodes were then stored at −20°C until further processing. For the macroscopic examination, the lymph nodes were thawed and cut into 1.0-mm thick slices and then examined for macroscopic lesions using a magnifying lens. Typical lesions of tuberculosis in red deer consist of purulent abscesses, though lesions can also be granulomatous (14, 21). If no lesions were found, the sample was considered negative. Lymph nodes from male animals aged older than 5 years were

TABLE 1 Number of samples collected from different wild ruminants and badgers for the risk-based approach.

	Red deer	Roe deer	Chamois	Ibex	Badger	Total
Male < 5 years	3	2	1	0	0	6
Male > 5 years	10	0	1	1	0	12
Female < 5 years	3	0	0	0	0	3
Female > 5 years	7	0	0	0	0	7
Age and/or sex unknown	1	1	2	0	3	7
Total	24	3	4	1	3	35

TABLE 2 Number of samples collected from red deer for the random sampling approach.

	Male	Female	Sex unknown	Total
< 5 years	87	151	0	238
> 5 years	65	201	0	266
Age unknown	4	3	2	9
Total	156	355	2	513

not macroscopically examined in detail, since they were directly processed for mycobacterial culture.

2.3 Mycobacterial culture

Culture of mycobacteria was conducted for all lymph nodes with suspicious lesions and for all male animals aged older than 5 years. For the mycobacterial culture for each animal, all the received lymph nodes were pooled. First, the lymph nodes were cut into small pieces and homogenized using a disperser (T18 digital ULTRA-TURRAX®, IKA, Staufen, Germany). Following, decontamination of the homogenized lymph nodes was conducted to get rid of organic contamination and normal flora. The decontamination was carried out according to a protocol described by the WHO in the Laboratory Services in Tuberculosis Control Part III (22). For that purpose, 4% H_2SO_4 was added. After 15 min of incubation, it was neutralized by the addition of 1N NaOH. The sample was then washed with PBS and centrifuged for 15 min at 3000 g. The sediment was resuspended in PBS buffer and streaked on a rigid growth medium for mycobacterial culture (Löwenstein–Jensen and Stonebrink media, Artelt–Enclit, Rötha OT Oelzschau, Germany) and additionally to the liquid mycobacteria growth indicator tube (MGIT™, Becton Dickinson, Allschwil, Switzerland), where BD MGIT™ growth supplement and BD MGIT™ PANTA™ antibiotic mixture were added. To support the growth of *M. bovis* and MAP, pyruvate (Carl Roth AG, Arlesheim, Switzerland) and mycobactin (Innovative Diagnostics, Grabels, France) were added to the MGIT™.

The mycobacterial cultures were incubated at 37°C for 7 weeks. The Löwenstein–Jensen medium and Stonebrink medium were checked for growth once a week. The MGIT™ was incubated in the BD BACTEC™ MGIT™ 320 system, which conducts hourly fluorescence measurements. If the BD BACTEC™ MGIT™ 320 system measured a positive result, Ziehl–Neelsen staining was performed to confirm the growth of acid-fast bacilli. Afterward, the samples were subcultured on a Middlebrook 7H10 solid growth medium (Becton Dickinson, Allschwil, Switzerland), supplemented with an in-house PANTA antibiotic mixture. After the growth of mycobacteria, real-time PCR (*artus M. tuberculosis* RG PCR Kit, Qiagen, Hilden, Germany) was performed to exclude the presence of MTBC. MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA) was used for the identification of mycobacteria. In two cases, MAP was detected, which was confirmed with PCR (ID Gene™ Paratuberculosis Duplex, Innovative Diagnostics, Grabels, France). The two MAP strains were further characterized by MIRU-VNTR, PCR, and restriction enzyme digestion for the identification of C-type or S-type strain, as previously described (23).

2.4 Histology

Different tissues from six animals with macroscopic lesions were fixed in 4% formalin (Table 3). The following day, formalin-fixed tissues were trimmed and embedded in paraffin, and histological tissue sections of 2–3 µm thickness were cut and stained with hematoxylin and eosin (HE). Slides were then assessed under a light microscope.

3 Results

3.1 Macroscopic evaluation of the lymph nodes

In the risk-based approach, seven animals (two red deer, one roe deer, three chamois, and one badger) had macroscopic lesions in their lymph nodes or other organs, as shown in Table 3. One of these animals (chamois) was male and aged older than 5 years. In the random sampling group, three female red deer had purulent lesions in their lymph nodes. None of the other animals had any macroscopic lesions.

3.2 Mycobacterial culture

In the risk-based approach, mycobacterial culture was carried out in 20 out of 35 samples (57.1%). In total, 12 of these animals were male animals aged 5 years or older (Table 1; 10 red deer, 1 chamois, and 1 ibex). Additionally, the mycobacterial culture

TABLE 3 Animals with macroscopic lesions in their lymph nodes or other organs and results from histology and mycobacterial culture.

Sampling approach	Animal	Macroscopic lesions	Histology	Result of the mycobacterial culture
Random sampling	Red deer, f > 5 years	Pus-filled lymph node	-	Negative
	Red deer, f > 5 years	Pus-filled palatine tonsil	Moderate multifocal suppurative necrotizing tonsillitis	Negative
	Red deer, f > 5 years	Pus-filled lymph node	Slight eosinophilic lymphadenitis	Positive for *M. nonchromogenicum*
Risk-based sampling	Red deer, sex and age unknown	Nodules under the skin	-	Negative
	Red deer, f < 5 years	Pus-filled kidney	Renal carcinoma	Negative
	Roe deer, sex and age unknown	Lung abscess	-	Negative
	Chamois, sex and age unknown	Abscess on the head, abscessing pneumonia	Multifocal to diffuse granulomatous and eosinophilic pneumonia with parasite cut sections	Negative
	Chamois, sex and age unknown	Inconclusive macroscopic evaluation of the lymph nodes, lesions in the lungs	-	Negative
	Chamois, m > 5 years	Suspicious lesions in lymph nodes, lesions in the lungs	Multifocal granulomatous and eosinophilic pneumonia with parasite cut sections	Positive for *M. vaccae*
	Badger, sex and age unknown	Calcifications in the prescapular lymph nodes, lesions in the spleen, liver, kidney, and lungs, positive for canine distemper virus	Multifocal granulomatous and eosinophilic pneumonia with parasite cut sections	Positive for *M. avium*

of lymph nodes with macroscopic lesions of one roe deer, two chamois, one badger, and two red deer was performed (Table 3). Furthermore, the lymph nodes of two badgers without macroscopic lesions were included in the culture, as badgers do not always show typical lesions in their lymph nodes (24, 25). Positive cultures for NTM were detected in six samples (6 of 20, 30%; Table 4). Only two animals with macroscopic lesions had a positive result in the mycobacterial culture (Table 3). One of them, a male chamois aged older than 5 years with macroscopic lesions in the lymph nodes and pneumonia with parasites, was positive for *M. vaccae*. The other culture-positive animal with lesions was a badger, which had lesions in the lymph nodes and various organs and was also found to be infected with the canine distemper virus and lungworms. This badger was positive for *M. avium*. The other two badgers, which did not have any lesions, were also found to be positive for *M. avium* (Table 4). There were two more culture-positive animals, namely, two male red deer aged older than 5 years without any macroscopic lesions. These two animals were infected with *M. diernhoferi* and a mixed infection with two different NTM, respectively.

In the random sampling group, 68 out of 513 samples (13.3%) were tested with mycobacterial culture. In total, 65 of the samples belonged to male animals older than 5 years, without any obvious macroscopic lesions in their lymph nodes. Overall, 18 of these male animals had a positive culture. Only one of the female animals with macroscopic lesions in its lymph nodes had a positive culture for NTM (*M. nonchromogenicum*). Altogether, a positive culture for NTM was detected in 19 samples (19 of 68, 27.9%; Table 4).

Overall, 548 samples were analyzed, of which 88 samples (16.1%) were tested with the mycobacterial culture and 25 samples were positive (25 of 88, 28.4%). The different species identified are listed in Table 4. No animal had a positive culture for the *Mycobacterium tuberculosis* complex. Overall, *M. vaccae* was identified most often (8 of 25, 32%). The second most identified NTM was *M. avium* (7/25, 28%), which was further identified as *M. avium* ssp. *hominissuis* in three cases and MAP in two cases. Both MAP-positive animals were male red deer from the random sampling group, aged 8 and 13 years, respectively. The samples from these two animals were taken in the slaughterhouse. Neither of these two animals had any macroscopic lesions in their head lymph nodes or any other signs of disease. However, the shooting sites of the two animals were located in Grisons near Alpine pastures, which were used for summering of cattle. Both MAP strains were identified as C-type belonging to the INMV1 profile. Furthermore, in four culture-positive cases, the species could not be identified due to poor growth of the mycobacteria. These strains were classified as NTM. Additionally, in one of these cases, there was a mixed infection with two different NTM.

3.3 Histology

In all tissue samples histologically examined (Table 3), no lesions interpreted as induced by NTMs could be discerned. In the risk-based approach, four animals were histologically examined. Three animals, whose lung tissue was assessed, had pneumonia induced by lungworms. Two of these animals were also positive in the mycobacterial culture, namely, for *M. vaccae* and *M. avium*, respectively. One animal with a renal carcinoma interpreted as an incidental finding had no histological lesions in the lymph node and was negative in the mycobacterial culture.

TABLE 4 Samples submitted to mycobacterial culture with the identified mycobacterial species.

Sampling approach	Animal species	Number of animals tested with mycobacterial culture	Number of mycobacterial culture positive samples	Mycobacteria identified	Number of strains identified
Random sampling	Red deer	68	19	*M. avium* ssp. *paratuberculosis*	2
				M. avium ssp. *hominissuis*	2
				M. diernhoferi	1
				M. nonchromogenicum	2
				M. porcinum	1
				M. terrae complex	1
				M. vaccae	7
				NTM	3
Risk-based sampling	Red deer	12	2	*M. diernhoferi*	1
				NTM (mixed-infection)	1
	Roe deer	1	0	-	-
	Chamois	3	1	*M. vaccae*	1
	Ibex	1	0	-	-
	Badger	3	3	*M. avium*	2
				M. avium ssp. *hominissuis*	1
	Total	20	6		
Total		88	25		

In the group from the random sampling, histology was carried out in two animals. One animal had a suppurative necrotizing tonsillitis, with a negative mycobacterial culture. The other animal had a slight eosinophilic lymphadenitis, which was most likely induced by parasitic infestation. This animal was also positive for *M. nonchromogenicum*.

4 Discussion

Our study is the first to describe the isolation of MAP from the head lymph nodes of red deer in Switzerland. Furthermore, the isolation of various NTM provides an overview of different NTM species found in Swiss wild ruminants and badgers. In concordance with other studies, no MTBC was detected.

MAP was isolated from the head lymph nodes of two red deer. To the best of our knowledge, this is the first isolation of MAP from red deer in Switzerland. However, there is a reported case of MAP in a roe deer from Switzerland, which had severely enlarged lymph nodes and a case history of severe diarrhea (26). Another study from Switzerland that tested fecal samples from different wild ruminants was not able to cultivate MAP, suggesting only a low occurrence of MAP in Swiss wild ruminants (9). Recently, we detected another MAP-positive red deer in Switzerland (data not shown), which supports the low occurrence of MAP and also suggests that the other two MAP-positive red deer were not just a coincidental finding. Generally, the MAP prevalence in wildlife in other countries is also quite low, though the prevalence can be rather high in some countries, for example in Northern Italy and Spain (8, 27–29). However, the diagnosis of paratuberculosis is complicated by its long incubation period, the intermittent shedding of MAP in feces, and the lack of completely reliable diagnostic tests (30, 31), which might lead to an underestimation of the MAP prevalence. It is suggested that cattle are the source of infection for wild animals, although transmission from wild animals to cattle is possible as well (6). Interestingly, the two MAP-positive red deer in our study were both shot close to Alpine pastures used for grazing of cattle during summer. Thus, it is possible that the pastures are contaminated with MAP and pose a source of infection for red deer. The two MAP strains in our study were identified as C-type strains; the pattern found by MIRU-VNTR was INMV1. INMV1 is the predominantly found profile in Swiss cattle (23). Neither of the two MAP-positive animals had any clinical signs of disease or any macroscopic lesions. Other studies also found that the majority of MAP-infected red deer are asymptomatic and without macroscopic lesions (7, 28). Hence, it is possible that we missed some MAP-positive animals in our study since we did not conduct mycobacterial culture from all sampled animals. Furthermore, young red deer aged 8–15 months seem to be more susceptible to MAP infection than older animals and are thus more likely to develop clinical signs (7, 32). This might also explain the lack of clinical signs in the two MAP-positive red deer from our study, since the animals were already 8 and 13 years old, respectively. While vertical transmission of MAP is possible, the typical route of infection is fecal–oral. After ingestion, the pathogen is found within macrophages in the submucosa of the ileocecal area

and its surrounding lymph nodes (1). Hence, typical samples for the diagnosis of MAP include feces, intestinal lymph nodes, the ileocecal valve, or intestines (8). However, in our study, we detected MAP in the head lymph nodes. A likely explanation for this would be that the head was the primary entry site of MAP (33), as head lymph nodes are also described as the primary site of infection for other mycobacteria (14).

Apart from MAP, we detected various NTM in different wild ruminants and badgers. The most often identified species was *M. vaccae*, which we detected in red deer and chamois. In Switzerland, *M. vaccae* was detected in wild boar before (5). In other countries, *M. vaccae* was also isolated from red deer (2, 3, 34). For *M. vaccae,* there is no histological evidence for pathogenicity in deer (34). In our study, most animals infected with this pathogen did not show any lesions in their lymph nodes, which supports the non-pathogenic nature of this agent. However, a chamois infected with *M. vaccae* did have suspicious lesions in its lymph nodes. While we cannot rule out other pathogens as the cause of these lesions, it is possible that *M. vaccae* can cause a disease under certain conditions. Similar results were observed for *M. nonchromogenicum*, for which only one animal showed macroscopic lesions, while, according to de Lisle, there is also no histological evidence for pathogenicity in deer (34). The second most identified NTM in our study was *M. avium*, which could be further specified as *M. avium* ssp. *hominissuis* in three cases and was found in red deer and badgers. In Swiss wild boars, *M. avium* ssp. *hominissuis* was the most often identified NTM (5); thus, it seems to be widespread among wildlife in Switzerland. Furthermore, *M. avium* ssp. *hominissuis* was also frequently detected in Slovenia (3). In our study, only one of the badgers infected with *M. avium* had lesions in the lymph nodes and other organs. However, this badger also tested positive for the canine distemper virus, which might have weakened the immune system, thus making the badger more susceptible to the mycobacterial infection. According to other studies, *M. avium* can be found in animals with and without lesions (3, 5, 35). In our study, animals infected with all other NTM species did not show any macroscopic lesions. Hence, it seems that most NTM are not pathogenic for wildlife, although some NTM might cause disease under certain conditions. This is similar to the findings in humans, where many NTM are considered opportunistic pathogens that mainly cause diseases in immunocompromised people (36). However, more studies are needed to determine the pathogenic potential of different NTM in animals. The prevalence and variety of different NTM species detected in Swiss wild boars were much higher than in our study, and apart from *M. porcinum* and MAP, all NTM species isolated in our study were detected in Swiss wild boars before (5). Thus, it seems likely that the prevalence and variety of NTM species found in wildlife are higher than we detected, and that the same NTM species can be found in different wild animals. Other countries also reported that a high variety of NTM was found in different wild animals, and apart from *M. porcinum*, all NTM species isolated in our study were detected in other countries before (2, 3, 34, 35). Furthermore, there are most likely still unknown NTM species, which could explain, why we could not identify all NTM in our study. This variety of NTM found in animals can be explained by the occurrence of NTM in the environment. Through contact with different sources of NTM, such as soil, dust, or water, animals can get infected (36).

As a conclusion, various NTM can be found in different wild animals in Switzerland. It seems that most of these NTM are not obligate pathogens, though some might act as opportunistic pathogens.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because the animals investigated in this study were either shot during regular hunting or found dead from a natural cause.

Author contributions

JL: Formal analysis, Visualization, Writing—original draft. UF: Data curation, Investigation, Methodology, Writing—original draft. CP: Conceptualization, Resources, Writing—review & editing. MH: Data curation, Formal analysis, Investigation, Writing—original draft. SaS: Conceptualization, Methodology, Project administration, Resources, Supervision, Writing—review & editing. SiS: Data curation, Formal analysis, Investigation, Writing—review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors would like to thank the veterinarians, the hunters, and the Cantons of Grisons and St. Gallen and the Principality of Liechtenstein for the collection and provision of the samples.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 25 January 2024
DOI 10.3389/fvets.2024.1303096

OPEN ACCESS

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RECEIVED 27 September 2023
ACCEPTED 10 January 2024
PUBLISHED 25 January 2024

CITATION
Filippi A, Garbarino C, Nava M, Russo S, Soares Filipe JF, Bianchi A, Corlatti L, Gugiatti A, Buccheri Pederzoli C, Pigoli C, Pedrotti L, Arrigoni N, Ricchi M, Bertoletti I and Luzzago C (2024) Active surveillance of paratuberculosis in Alpine-dwelling red deer (*Cervus elaphus*). *Front. Vet. Sci.* 11:1303096. doi: 10.3389/fvets.2024.1303096

Active surveillance of paratuberculosis in Alpine-dwelling red deer (*Cervus elaphus*)

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Paratuberculosis (Johne's disease) is a globally widespread infectious disease affecting domestic and wild ruminants, caused by *Mycobacterium avium subsp. paratuberculosis* (MAP). The bacterium is excreted in the feces and is characterized by high environmental resistance. The new Animal Health Law (Regulation EU 2016/429) on transmissible animal diseases, recently in force throughout the European Union, includes paratuberculosis within the diseases requiring surveillance in the EU, listing some domestic and wild Bovidae, Cervidae, and Camelidae as potential reservoirs. Taking advantage of a culling activity conducted in the Stelvio National Park (Italy), this study investigated MAP infection status of red deer (*Cervus elaphus*) between 2018 and 2022, and evaluated the probability of being MAP-positive with respect to individual and sampling-level variables. A total of 390 subjects were examined macroscopically and tested for MAP, using different diagnostic tools: IS900 qPCR, culture, histopathology, and serology. Twenty-three of them were found positive for MAP by at least one test, with an overall prevalence of 5.9% (95% CI 4.0–8.7), that, respectively, ranged from 12.4% in the first culling season to 2.0 and 2.1% in the 2019–2020 and 2021–2022 culling seasons. Quantitative PCR assay on ileocecal valve and mesenteric lymph nodes detected the highest number of MAP positive animals. The results of the study showed the increased probability of being MAP-positive with increasing age and that red deer with lower body mass values were more likely to be infected with MAP. Overall, the absence of signs of clinical paratuberculosis and gross lesions together with the low level of shedding witness early phases of the disease among the positive red deer and support an improvement of the paratuberculosis status of this population, as shown by the decreased prevalence of the disease over the years.

KEYWORDS

paratuberculosis, red deer, wild animal, prevalence, surveillance, Italy

1 Introduction

Paratuberculosis is a worldwide distributed chronic intestinal infection caused by *Mycobacterium avium subsp. paratuberculosis* (MAP). MAP is a multi-host pathogen, highly resistant to severe environmental conditions (1). Many species are susceptible to MAP infection (2), but ruminants are the most susceptible, showing well-documented evidence of disease (3). By excreting MAP in feces, ruminants can contaminate the environment and infect other animals through fecal-oral route (4). Cattle, sheep, and goats are commonly affected, and wild ruminants, mainly cervids, are considered reservoirs, sharing MAP isolates with domestic livestock (5–8). Where domestic and wild species co-occur, interspecific transmission of disease may happen, also favored by environmental contamination by infected individuals (3, 9).

In Italy, the first reported case of paratuberculosis in wild ruminants was identified in red deer (*Cervus elaphus*) in the southern-Tyrolean part of the Stelvio National Park, in the Province of Bolzano (10). Afterward, the prevalence of MAP was estimated by molecular methods in red deer in several Alpine areas, showing values between 18.6 and 66.2% (11, 12), and an age-related pattern, with the highest prevalence in calves and yearlings (13). The Italian Ministry of Health has implemented paratuberculosis surveillance in cattle since 2013, recently extended to goat and sheep, on a voluntary basis, and a risk-based certification (14). The plan includes diagnostic screening, passive clinical surveillance on domestic ruminants, and adoption of biosecurity and management measures. Moreover, extensive livestock production, where domestic and wildlife animals interact, should consider both populations for disease control. The Animal Health Law (Regulation EU 2016/429) on transmissible animal diseases, recently in force throughout the European Union, includes paratuberculosis within the diseases that require surveillance in the EU, listing some domestic and wild Bovidae, Cervidae, and Camelidae as potential reservoirs (2).

The knowledge of the epidemiological role of wild animals is pivotal in order to progressively mitigate the risk of paratuberculosis infections in domestic animals. In Alpine areas, the risk of cross-transmission between domestic and wild ruminants is of particular concern during summer, as the interactions on pastures may facilitate the establishment of a multi-host system (15–17). The wildlife-livestock interactions have also been reported around supplementary feeding in wintertime (18). Taking advantage of a population of red deer subjected to a culling program in the Stelvio National Park (SNP), the aims of this study were: (i) to assess the temporal variation of MAP prevalence over three culling seasons and (ii) evaluate relationships between the probability of being MAP-positive and individual and sampling-level variables such as age, sex, body mass or individual conditions.

2 Materials and methods

2.1 Study area and population

The study area was located in the Lombardy sector of the SNP (Central Italian Alps) and ranges between 1,200 and 2,400 m a.s.l with an extension of approximately 2,600 ha, corresponding to the red deer wintering site. The red deer study population includes approximately 1,200 animals according to the annual counts, with a density of about 28 ind./km^2 in the wintering site (19). Supplementary feeding was not applied in the study area. Other wild ruminants in the area include roe deer (*Capreolus capreolus*), Alpine chamois (*Rupicapra rupicapra*), and Alpine ibex (*Capra ibex*), which are present in very low densities and overlap with red deer in marginal areas. Furthermore, herds of cattle and small domestic ruminants share Alpine pastures with wild ruminants from mid-June until mid-September.

Given the impact on forest regeneration and biodiversity, since 2011 the Park has decided to reduce red deer density through culling in the wintering sites. The culling plan was authorized by the Italian Institute for Environmental Protection and Research (Prot. 48585/T-A25-Ispra).

2.2 Samples and data collection

Red deer were culled by authorized hunters in the cold season, between November and February, under the supervision of the Park Authority. The present study was carried out over three culling seasons (2018–2019, 2019–2020, and 2021–2022), as the COVID-19 pandemics caused the suspension of culling in 2020–2021. Considering that the culling activity was aimed at reducing the number of red deer inside the Park, culling has been focused on the young age classes and on females, rather than on mature males. However, both the age distribution and the sex distribution remain consistent across years, with the latter varied from a ratio of males-females of 1:1.7 in the first year to 1:1.3 in the third year (Supplementary Figures 1, 2). Culled red deer must underwent to veterinary inspection in an authorized game meat processing center, following EU Regulation for game meat hygiene, making it possible to sample individuals. Within a few hours after culling, animals were brought to the check point with entire gastrointestinal tract in the first culling season, whereas ileocecal valve and mesenteric lymph nodes were directly sampled in the field during the second and third culling seasons. Individual information including age, sex, body mass (the values refers to the weight of eviscerated animals) and kidney fat index (KFI) were collected to investigate the relationship with MAP status. A macroscopic inspection was performed on all individuals to detect signs of illness including poor body condition, diarrhea, and gross lesions. MAP status was investigated through: (i) tissue samples (361 samples of ileocecal valves and mesenteric lymph-nodes) stored at −20°C (additionally, a portion of tissue samples was stored in 10% formalin in 2018–2019 and 2019–2020); (ii) feces ($N = 189$) directly collected from rectum and stored at −20°C; (iii) blood serum ($N = 315$) from major vessels, obtained through centrifugation at $400 \times g$ for 10 min of the whole blood and then stored at −20°C.

2.3 Real-time quantitative PCR on tissues and feces

To detect MAP-positive animals from tissue and feces, DNA was extracted from 25 mg of individual pools of ileocecal valves and mesenteric lymph-nodes (11) and from 3 ± 0.5 g of feces, as reported in Russo et al. (20). Quantitative PCR was performed on eluted DNA targeting IS900 sequence (20). Results were expressed by cycle of quantification (Cq) values, considering Cq values of two

replicates of the same sample. In particular, a sample was considered positive if both replicates resulted ≤36 Cq, negative if both replicates were > 36 and inconclusive if one replicate resulted ≤36 and one >36. Analysis of inconclusive samples was repeated and if the inconclusive result was confirmed, the sample was considered negative.

2.4 Bacteriology

To further confirm the presence and viability of MAP circulating in the red deer population, samples positive to qPCR were submitted to culture. The bacteriological method was performed on 3 g of the individual pool of ileocecal valves and mesenteric lymph-nodes ($N = 214$), according to a method already described by Savi et al. (21). Briefly, the mucosa and lymph node parenchyma (if present) were decontaminated with Hexadecylpyridinium Chloride (HPC, 0.75% in sterile distilled water), washed with Phosphate Buffered Saline (PBS) and then incubated in Herrold's egg yolk medium (HEYM), containing 2 mg of mycobactin J/L, supplemented with Chloramphenicol (30 mg/L) (HEYM/CAF) or with Nalidixic acid (50 mg/L), Vancomycin (50 mg/L) and sodium pyruvate (4 g/L) (HEYM/ANV). Results are reported considering the number of colony-forming units (CFU) and are expressed as: +, weakly positive (1–9 CFU/slant); ++, moderately positive (10–49 CFU/slant); +++, strongly positive (50–99 CFU/slant); and ++++, very strongly positive (> 99 CFU/slant). Suspected colonies were confirmed by qPCR targeting F57, Ziehl–Neelsen staining and Mycobactin dependency (7).

2.5 Histopathology

Formalin-fixed samples of the ileocecal valve and mesenteric lymph nodes were paraffin-embedded for histopathological examination. Two serial 3 µm thick sections were cut from each obtained paraffin block. One section was stained with hematoxylin–eosin. If microscopic lesions consistent with paratuberculosis infection were observed (22), the second section underwent Ziehl-Neelsen staining.

2.6 Serology

Sera were tested for MAP antibody presence through an Enzyme-Linked ImmunoSorbent Assay (ELISA) commercial kit (ID Screen® Paratuberculosis Indirect and ID Confirmation® Paratuberculosis Indirect, both ID VET, Montpellier, France) based on procedure steps indicated in the manufacturer's protocol. The test employed has a specificity over 99%, thanks to a pre-adsorption step with *Mycobacterium phlei* and a screening test followed by a confirmation one. The test provides firstly a monocupola test, used to discriminate positive and inconclusive samples from negative ones; inconclusive ($0.6 < S/P$ ratio < 0.7) and positive ($S/P \geq 0.7$) samples to the screening test were then submitted to confirmatory test, where a bicupola kit was used in order to verify the specificity of the reaction. In more details, each sample resulted suspected positive or inconclusive to the first assay, was tested in duplicate; one duplicate was tested on a non-adsorbed well and the other on a well adsorbed with MAP antigen. Samples with a S/P ratio of 0.7 or above in the confirmation test were considered positive.

2.7 Statistical analysis

To investigate possible associations between MAP positivity and individual or sampling-level variables [sex, age class (calves, yearlings and adults of 2+ years), body mass, KFI and density], we used Generalized Linear Models (GLMs). The analyses were conducted with R (R Core Team 2022) in RStudio (Rstudio Team 2022). An individual was defined as MAP positive if at least one of the diagnostic tests (ELISA or PCR) resulted positive. MAP infection status [either 1 (positive) or 0 (negative)] was fitted as a binary response variable, while sex, age class, body mass, KFI, and density, were fitted as explanatory variables. Body mass and KFI were adjusted to the first day of culling by fitting quadratic linear models between any given value of the target variable and Julian date, within each year and for different age-classes (calves, yearlings, and adults) (23). Although deer are social animals organized in groups, the sexes stay mainly divided. This social behavior and population density may contribute to a possible association on the probability of MAP-positivity. The global model included the interaction of sex with individual variables, and was of the form:

$$\text{MAP infection status} \sim \text{Year} + \text{Sex} \times (\text{Age class} + \text{Body mass} + \text{KFI} + \text{Density})$$

All explanatory variables were scaled prior to model fitting, to make estimates comparable and avoid issues of collinearity in presence of interactions; density was not kept in the final model due to collinearity with other variables. The package "DHARMa" (24) was used for residual diagnostics. Starting from the global model, we selected a simpler structure using an AIC-based (Akaike Information Criterion) stepwise algorithm with the function "stepAIC" in the package "MASS" (25) and the marginal effects were plotted with the function "visreg" (26).

3 Results

Overall, out of the 390 individuals tested, 23 were found positive for MAP by at least one test, with a prevalence of 5.9% (95% CI 4.0–8.7), ranging from 12.4% (95% CI 8.0–18.8) in the first culling season to 2.0% (95% CI 0.5–7.0) and 2.1% (95% CI 0.7–5.9) in the 2019–2020 and 2021–2022 culling seasons, respectively. Apparent paratuberculosis prevalence in relation to culling season and other explanatory variables is reported in Table 1. MAP positivity was detected both in calves (≤1-year-old) and adults. During the inspection at the control center, no signs of clinical paratuberculosis or gross lesions at post-mortem examinations were detected in any culled red deer. Details about positive samples, diagnostic test results and type of biological specimen (tissues, feces, and serum) are summarized in Table 2. Overall, out of 23 MAP positive individuals, 19 were identified by qPCR assay carried out on tissue samples. The remaining four MAP positive animals were detected by serology, showing an S/P ratio > 1.7. None of the MAP positive animals showed

10.3389/fvets.2024.1303096

TABLE 1 Paratuberculosis apparent prevalence according to culling season, age, sex, and type of sample of the Stelvio National Park.

Variable	Category	Positive/No. of tested animal (%)	Positive/No. of type of test (%)				
			qPCR tissue	qPCR feces	Culture tissue	Serology	Histopathology
Culling season	2018–2019	18/145 (12.4)	15/133 (11.3)	3/99 (3.0)	1/130 (0.8)	5/121 (4.1)	3/121 (2.5)
	2019–2020	2/100 (2.0)	1/84 (1.2)	0/33 (0)	1/84 (1.2)	1/62 (1.6)	0/54 (0)
	2021–2022	3/145 (2.1)	3/144 (2.1)	1/57 (1.8)	1/3*	1/132 (0.8)	n.p.**
Age	<1-year-old	4/126 (3.2)	4/112 (3.6)	1/64 (1.6)	1/70 (1.4)	0/103 (0)	0/54 (0)
	1 year old	3/62 (4.8)	3/60 (5.0)	0/27 (0)	2/30 (6.7)	1/49 (2.0)	1/27 (3.7)
	≥2 years old	16/202 (7.9)	12/189 (6.3)	3/98 (3.1)	0/114 (0)	6/163 (3.7)	2/94 (2.1)
Sex	Female	18/223 (8.1)	15/212 (7.1)	3/116 (2.6)	1/128 (0.8)	6/177 (3.4)	3/111 (2.7)
	Male	5/167 (3.0)	4/149 (2.7)	1/73 (1.4)	2/86 (2.3)	1/138 (0.7)	0/64 (0)
Total		23/390 (5.9)	19/361 (5.3)	4/189 (2.1)	3/214 (1.4)	7/315 (2.2)	3/175 (1.7)

*performed on qPCR positive on tissue. ** not performed.

TABLE 2 Red deer testing positive to at least one diagnostic test.

ID	Culling data	Sex	Age (years)	qPCR tissue	qPCR feces	Culture* tissue	Serology	Histopathology
1,067	2018-12-08	f	5.5	+	–	–	–	–
861	2018-12-15	f	2.5	+	+	–	+	+
959	2018-12-15	f	3.5	–	n.a.	–	+	–
1,047	2018-12-16	m	0.5	+	–	–	–	–
1,012	2018-12-18	f	12.5	+	–	–	–	–
1,147	2018-12-18	f	11.5	+	–	–	–	–
1,018	2018-12-20	m	0.5	+	–	–	–	–
986	2019-01-08	f	10.5	+	–	–	–	–
916	2019-01-22	f	0.5	+	–	–	–	–
996	2019-01-22	f	1.5	+	n.a.	–	–	–
1,049	2019-01-22	f	4.5	+	+	–	–	+
1,167	2019-01-22	f	2.5	+	–	–	–	–
1,168	2019-01-22	f	5.5	+	–	–	–	–
1,173	2019-01-22	f	1.5	+	–	+	+	+
1,174	2019-01-22	f	12.5	+	+	–	–	–
1,058	2019-02-09	f	7.5	–	–	–	+	–
1,126	2019-02-09	f	12.5	+	–	–	–	–
1,090	2019-02-12	f	9.5	–	–	–	+	–
1,234	2019-12-05	m	1.5	+	n.a.	+	n.a.	–
1,332	2020-01-16	m	2.5	n.a.	n.a.	n.a.	+	n.a.
1,231	2021-11-30	f	5.5	+	–	–	–	n.a.
1,118	2021-12-12	f	2.5	+	–	–	+	n.a.
1,225	2022-02-12	m	0.5	+	+	+	–	n.a.

n.a., specimen not available. *The number of colony-forming units (CFU) and are expressed as: $^{+}$ (weakly positive: 1–9 CFU/slant); $^{++}$ (moderately positive: 10–49 CFU/slant); $^{+++}$ (strongly positive: 50–99 CFU/slant); and $^{++++}$ (very strongly positive: > 99 CFU/slant).

positivity to all performed tests. Specifically, 15 out of 23 MAP positive samples were detected only by one test (12 by qPCR and 3 by serology), while the remaining animals resulted positive to both PCR and serology. Notably, qPCR assay performed on feces tested positive only in four out of 19 qPCR MAP positive on tissues. MAP isolation by cultural assay was achieved only in three samples out of 22 tested, all of them resulting weakly positive (1+, 1–9 CFU/slant) (Table 2); they were all collected from young animals.

Considering histopathology, during the 2018–2019 season, out of 145 culled red deer, 121 underwent histopathological examination. In

three animals, diffuse severe chronic granulomatous enterocolitis with Langhans-type multinucleated giant macrophages was found (Table 2); in the same subjects, granulomatous lesions were also found in the mesenteric lymph node. Ziehl-Neelsen staining revealed numerous intralesional acid-fast bacilli in these subjects. Notably, these three animals resulted positive to qPCR by tissues but showed a non-homogeneous trend to respect to the other assays employed. In the 2019–2020 season, 54 out of 100 culled red deer were subjected to histopathological investigations. None of the animals showed lesions consistent with paratubercular infection. No red deer culled during the 2021–2022 season underwent histopathological examination.

The model selection procedure performed to investigate possible associations between MAP positivity and collected variables returned a simpler model structure with the effects of year, sex, age class, body mass, and KFI and also the interaction of sex with body mass. However, this interaction was not significant and was excluded from the final model. Individual probability of being positive to MAP significantly increased with older age classes (Table 3; Figure 1A), with an estimated probability of 0.7% in calves, 4.7% in yearlings and 20% in adults, resulting in very high increases in the odds-ratio. With respect to year of sampling, probability of MAP positivity was significantly greater in 2018–2019 than in the remaining years with an estimated probability of 20% in 2018–2019, 4.7% 2019–2020, and 3.9% in 2021–2022 (Table 3; Figure 1B). Accordingly, there was an 80% decrease in the odds of being MAP positive from 2018–2019 to 2019–2020, and a decrease of 18% in the odds from 2019–2020 to 2021–2022. Body mass was negatively related to MAP positivity, as individuals with low body mass values were more likely to be infected with MAP than individuals with greater body masses, with a decrease of 82% in the odds of being MAP positive, per increase of 1 k in weight (Table 3; Figure 1C). Regarding KFI, the relation with MAP positivity was not statistically significant. Statistical analyses have been carried out also considering only the samples positive to tissue qPCR, but the results obtained did not differ from those obtained previously.

4 Discussion

Surveillance for paratuberculosis has recently been introduced within the frame of European Animal Health Law in livestock and extended also to wild species. In this regard, paratuberculosis health status of red deer is important not only for the red deer population as such, but also for all species susceptible to the disease sharing the same habitat.

In the study area, the recovering of MAP field isolates with INMV1 profile has been described in culled red deer (11) and in environmental deer feces (27). This profile has been already described in Italian cattle population (7), supporting the hypothesis of possible transmission of MAP from cattle to red deer and vice versa. In this contest, the epidemiology of paratuberculosis was investigated in a red deer population in which the prevalence of paratuberculosis was previously assessed to be around 20% between 2011 and 2015 (11).

Our results showed an improvement of the infectious status of the population, with a decline of the prevalence over the years, until about 2% in the last culling seasons (2019–2020 and 2021–2022). These data are in line with the low apparent prevalence observed in the Lombardy region cow population, where Paratuberculosis is widespread (> 52% herds infected) but with low animal prevalence (1.3%) (28). In addition, despite MAP-positive animals were detected also in calves (≤1-year-old), the prevalence increased with age. This last result is in contrast with Galiero et al. (11) who reported a similar prevalence in young (up to 2 years old) and adult animals in the same area. Moreover, a previous investigation carried out in the Trentino sector of the Park, bordering our study area, showed higher MAP prevalence in young individuals (13).

A shift in the epidemiological pattern over the years, with a decreasing trend of MAP prevalence at the population level and in young animals, could be hypothesized. The results of the present study, specifically the increased probability of being MAP-positive with increasing age, supports this suggestion. On the other hand, the mortality induced by the disease itself, might have contributed to the infection decline. This phenomenon could have been exacerbated during the years of the study because of an increased snowfall (Supplementary Figure 3) which in turn, could have increased mortality due to winter starvation.

Notably, the qPCR assay performed on tissues (ileocecal valve and mesenteric lymph nodes) detected most of MAP positive individuals, while the test performed on feces detected MAP just in four out of 19 qPCR MAP positive on tissues. This result is in the line with what was reported in dairy herds (29), and could be related to the intermittent shedding of MAP in feces by infected individuals and to the amount of MAP excreted. The excretion varies according to the progression of the disease, being very low or null in the first phases of the infection (30). In fact, the histopathology performed in the present study showed lesions consistent with paratubercular infection in a low number of infected animals, of which only two were positive to qPCR by feces.

In relation to the high specificity and the low sensitivity of paratuberculosis tests used, we considered as positive an animal testing positive at least to one test. In our study we adopted a diagnostic parallel approach in order to increase the sensitivity of the detection. Despite the effort on histopathology, this method identified a low number of positive samples, probably also related to the limited size/area of tissue sampled. Moreover, our surveillance underlines the low sensitivity of serology and qPCR in feces, which are the methods most commonly used both in domestic and in wild ruminants (3, 29). Therefore, considering our results, for both active and passive surveillance, we recommend to analyze tissues by qPCR to define,

TABLE 3 Estimates of the final selected model.

Parameter	Log-Odds	SE	95% CI
(Intercept)	−4.59	0.82	[−6.34, −3.09]
Year [2019–20 vs. 2018–19]*	−1.62	0.78	[−3.51, −0.29]
Year [2021–22 vs. 2018–19]*	−1.83	0.68	[−3.39, −0.63]
Age class[2] [1 vs. 0]*	1.9	0.91	[0.04, 3.73]
Age class[2] [2+ vs. 0]*	3.5	0.98	[1.63, 5.54]
Body mass*	−1.69	0.49	[−2.73, −0.75]
KFI	0.48	0.25	[−0.02, 0.96]

The table shows for each parameter: the logarithm of the odds ratio (Log-Odds), the standard error (SE), and the 95% confidence intervals (95% CI). Age class in years: 0, 1, and 2+. *statistically significant.

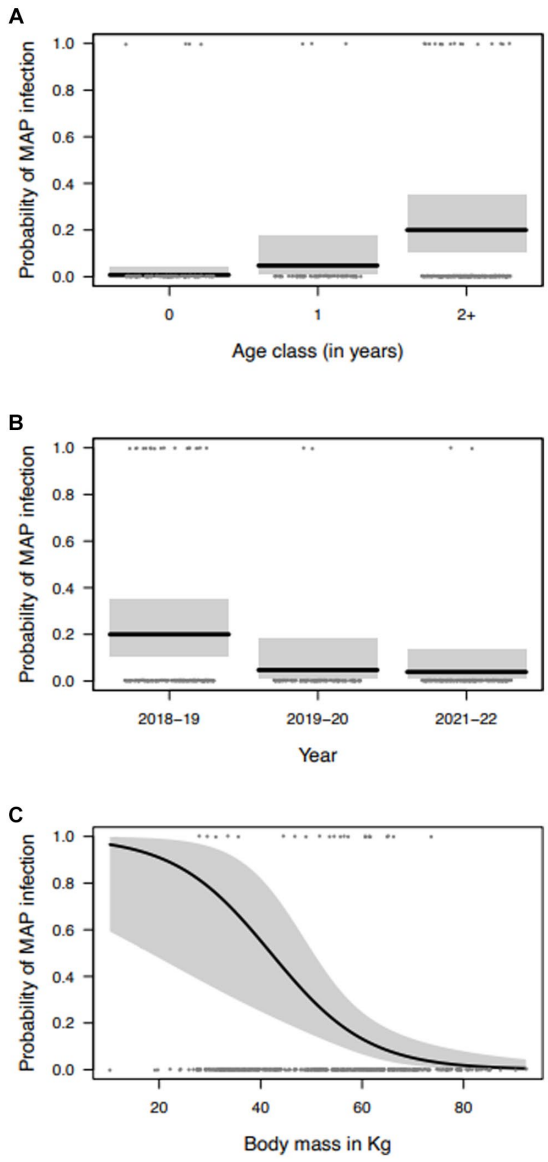

FIGURE 1
Marginal effects of the variables selected to explain the variation in the probability of MAP infection of red deer in the Stelvio National Park: **(A)** age class; **(B)** year of sampling; and **(C)** body mass (gray areas represent the 95% confidence intervals).

with a higher accuracy, the paratuberculosis health status of wild population.

With respect to the correlation between the other variables investigated and the probability of MAP infection, the fact that red deer with low body mass values were more likely to be infected with MAP is consistent with the pathogenesis and the characteristics of the disease, which may lead to nutrients malabsorption and, consequently, to body mass loss. This index may reflect longer term effects, while KFI is a body condition metric that should reflect animal conditions in the short term. With respect to the results about KFI, close to the significance level, the sample size was limited, therefore any interpretation requires caution. Further data should be collected in order to correlate potential effects of MAP on KFI.

Overall, the absence of signs of clinical paratuberculosis and gross lesions together with the low level of shedding (MAP CFU), supports the possible presence of animals in early phases of the disease and with subclinical infections. Notably, at this stage, the disease could be still controlled by the immune system of the animals, without a progression of the disease to the clinical stage. In particular, these considerations, together with the decreasing trend of MAP prevalence in young animals, witness an improvement of health status of this population. The mortality induced by the disease itself, together with culling program, could have contributed to a reduction of MAP spreading in the red deer population of this area.

In addition, the adoption of national guidelines for the control of bovine paratuberculosis led to a general improvement of livestock health status (28) in the Lombardy region, with a reduced risk of spreading on pastures and transmission to wildlife.

In accordance with European Animal Health Law, it will be important to continue paratuberculosis surveillance both on wild and domestic ruminants in order to increase knowledge useful for the management of both populations, thus reducing the risk of infection in Alpine habitats.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because no ethical approval was required for the present study and ethical statement is not applicable as sample collection from animals has been gathered after animals were culled for management purposes according to the official culling plan to reduce red deer density that has been authorized by Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), the Italian Ministry of Environment (Prot. 48585/T-A25-Ispra), in the Lombardy sector of the Park starting from 2011. Therefore, animals were not sacrificed for research purposes specific to this study.

Author contributions

AF: Data curation, Formal Analysis, Writing – original draft. CG: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft. MN: Data curation, Formal Analysis, Investigation, Writing – review & editing. SR: Formal Analysis, Writing – review & editing. JS: Formal Analysis, Writing – review & editing. AB: Investigation, Writing – review & editing. LC: Data curation, Formal Analysis, Writing – review & editing. AG: Data curation, Investigation, Writing – review & editing. CB: Investigation, Writing – review & editing. CP: Formal Analysis, Writing – review & editing. LP: Funding acquisition, Supervision, Writing – review & editing. NA: Supervision, Writing – original draft. MR: Supervision, Writing – original draft. IB: Investigation, Writing – review & editing.

CL: Conceptualization, Funding acquisition, Supervision, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The funds for this study were provided by Italian Ministry of Health, grant E54I19002810001 (Internal code PRC2019010) and by University of Milan (CUP G46D15001850005) Piano di Sostegno alla Ricerca 2017 (CL). MN was supported by a scholarship for promising graduates of University of Milan.

Acknowledgments

We thank the staff of the Stelvio National Park (Endrich Silvestri and Andrea Zanoli) for support in collecting data.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1303096/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Brief Research Report
PUBLISHED 31 January 2024
DOI 10.3389/fvets.2024.1361788

OPEN ACCESS

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RECEIVED 26 December 2023
ACCEPTED 15 January 2024
PUBLISHED 31 January 2024

CITATION
Gomez-Buendia A, Alvarez J, Bezos J, Mourelo J, Amado J, Saez JL, de Juan L and Romero B (2024) Non-tuberculous mycobacteria: occurrence in skin test cattle reactors from official tuberculosis-free herds. *Front. Vet. Sci.* 11:1361788. doi: 10.3389/fvets.2024.1361788

Non-tuberculous mycobacteria: occurrence in skin test cattle reactors from official tuberculosis-free herds

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Non-tuberculous mycobacteria (NTM) are considered a relevant cause of non-specific reactions to the most widely applied bovine tuberculosis (bTB) test, the intradermal tuberculin test. In order to establish which NTM species might act as a potential source of such diagnostic interference, a collection of 373 isolates obtained from skin test positive cows from 359 officially tuberculosis-free (OTF) herds, culled in the framework of the bTB eradication campaign in Spain, were identified at the species level through PCR and Sanger sequencing of the 16S rDNA, *hsp65* and *rpoB* genes. Of the 308 isolates for which a reliable identification was achieved, 32 different mycobacterial species were identified, with certain species being most represented: among *M. avium* complex members ($n = 142$, 46.1%), *M. avium* subsp. *hominissuis* (98; 69.0%) was the most abundant followed by *M. avium* subsp. *avium* (33, 23.2%), and *M. intracellulare* (7, 4.9%). Among non-MAC members ($n = 166$, 53.9%), *M. nonchromogenicum* (85; 27.6%) and *M. bourgelatii* (11; 5.6%) were the predominant species. In addition, mixed results were obtained in 53 isolates presenting up to 30 different genotypes, which could be indicative of new mycobacterial species. Our results represent a first step toward characterizing the diversity of NTM species that could interfere with official diagnostic tests for bTB eradication in Spain.

KEYWORDS

cattle, diagnosis, officially tuberculosis-free (OTF) herds, interference, non-tuberculous mycobacteria, tuberculosis, skin tests

1 Introduction

The genus *Mycobacterium* is very large, encompassing 196 different child taxa with validly published and correct names described (1). Mycobacteria can take on different roles: there are highly relevant animal and human pathogens such as *M. bovis* and *M. caprae*, members of the *Mycobacterium tuberculosis* complex (MTC) and the most common causative agents of bovine tuberculosis (bTB), while others like the non-tuberculous mycobacteria (NTM) are typically free-living microorganisms widely distributed in the environment. These NTM can be found in soil, water, dust, etc. (2), but in certain cases, usually linked to immunosuppressive processes, they can infect humans and animals and

act as opportunistic pathogens (3). In cattle, infection with NTM has not been traditionally associated with the presence of clinical signs [with the notable exception of *M. avium* subsp. *paratuberculosis* (*Map*), the causative agent of paratuberculosis], although the presence of granulomatous lesions as a consequence of infection has been described (4–7).

Since the beginning of the establishment of bTB eradication programs in different regions of the world in the early 1900s, several countries have been successful in the eradication of the disease (8). In others, however, despite all the efforts made, the disease is still endemic (9). Failure of these programmes has been partly attributed to the limitations in the sensitivity and specificity of existing diagnostic tests. Among factors compromising the performance of these tests, NTMs have been repeatedly linked to the occurrence of non-specific reactions in the single and comparative intradermal tuberculin tests (SIT and CIT), the main diagnostic tools used as the basis of eradication programs worldwide (10).

Most studies evaluating the role of NTM in bTB diagnostic problems have focused on those produced by *Map*, showing that it can affect both specificity and sensitivity (11–14). Nevertheless, NTM other than *Map*, also known as atypical or environmental, can also impact the reliability of bTB diagnostic tests due to the occurrence of cross-reactions in TB-free animals (15, 16), while their impact on the sensitivity of the tests remains to be quantified. Several studies have identified which species may be most commonly associated with these cross-reactions on bTB diagnostic tests in different settings, with *M. avium* subspecies and *M. nonchromogenicum* being the most commonly retrieved mycobacteria from bovine samples (15, 17–19). However, not all samples evaluated in these studies were from test positive animals or officially tuberculosis-free (OTF) farms. For instance, in Spain a recent study describing the presence of NTM in animals included only three reactor cattle from OTF herds, with other isolates originating from herds in which the disease was present, thus making the role of these bacterial species on the occurrence of cross-reactions unclear (19).

Therefore, the objective of this study was to identify the species associated with non-specific reactions detected in the framework of the bTB eradication campaign in Spain in a large cohort (n = 373) of skin test-reactor cattle from OTF herds located in areas with different prevalence of the disease. This information can be useful for better understanding the primary species affecting the specificity of skin tests for bTB and to assess the geographical variability in their occurrence.

2 Materials and methods

All NTM isolates available at the VISAVET Health Surveillance Center retrieved between 2011 and 2020 from skin test-positive cattle located in 359 OTF herds detected in the frame of the Spanish eradication program were evaluated. Isolates were either cultured directly at VISAVET from cattle samples collected at the abattoir and analyzed in the laboratory there or submitted by official veterinary laboratories located in two OTF regions (Asturias and Galicia, 72.1% of the herds), with the remaining sampled animals originating from different parts of the country (Figure 1). Veterinary laboratories submitted the isolates to VISAVET for identification once they had confirmed they did not belong to the *M. tuberculosis* complex except for the OTF region of Asturias, which mostly submitted isolates already identified as belonging to the *M. avium* complex (MAC).

According to the eradication program, when a skin test positive animal is identified in an OTF herd the animal must be culled within a maximum of 15 days and tissue samples are collected in the abattoir and submitted to an authorized laboratory in order to isolate or detect in the samples the presence of MTC members through bacteriology (20) and more recently direct PCR (21). Bacteriological analysis of these samples can occasionally lead to the identification of NTM species, which are identified as such in the laboratory according to the following protocol (Figure 2): once growth is observed, DNA is extracted and subjected to multiplex PCR for the detection of a *Mycobacterium*-specific DNA fragment (16S rDNA sequence) (22) and of a MTC-specific target (*mpb70* gene) (22). If the presence of a MTC member is not detected, another PCR aiming at a MAC-specific 16S rDNA (22) is performed, followed in case of a positive result by two PCRs aiming at the IS*901* and IS*1245* sequences associated with *M. avium* subsp. *avium* (*Maa*) (23) and *M. avium* subsp. *hominissuis* (*Mah*) (24). The available NTM strain collection included all isolates identified as *Mycobacterium* spp. based on the detection of the 16S rDNA *Mycobacterium* fragment but not belonging to MTC based on the absence of the *mpb70* gene that had been initially cultured at VISAVET or that were cultured in other authorized laboratories and later submitted to VISAVET for further identification. Isolates not belonging to MAC according to the methodology explained above were subjected to PCR amplification followed by Sanger sequencing of several targets: first, sequences of partial fragments of the 16S rDNA (22) and *hsp65* (Telenti fragment) genes (25, 26) were generated. Forward and reverse sequences were curated using BioEdit software (27) and combined to yield a consensus sequence that was then screened using the NCBI Basic Local Alignment Search Tool (BLAST) to identify the bacterial species. An identification was considered reliable if sequence similarity and coverage with a target was >99 and 100%, respectively (28). If the 16S rDNA/*hsp65* analysis yielded a non-reliable identification (due to low similarity/coverage and/or disagreement in the species identified through each target), amplification and sequencing of fragments of the *rpoB* gene (29) and then the 3′ region of the *hsp65* gene (30) was conducted and analyzed as described above. Full workflow is shown in Figure 2. Prior to bacteriological culture, samples were evaluated for the presence of macroscopial lesions compatible with bTB (31).

All primers used are shown in Supplementary Table 1. Sequencing was performed by STABvida (Lisbon, Portugal).

3 Results

Overall, 373 isolates recovered from positive cattle located in 359 OTF herds across 34 provinces (15 Autonomous Communities) were included in the study (Figure 1). Of these, 194 (54.0%) were beef herds, 150 (41.8%) were dairies, eight (2.2%) were fattening units, and seven (1.9%) were bullfighting herds. Of the 373 isolates, we were able to achieve a reliable identification in 308 (82.6%). Among these, 166 (53.9%) were classified as non-MAC

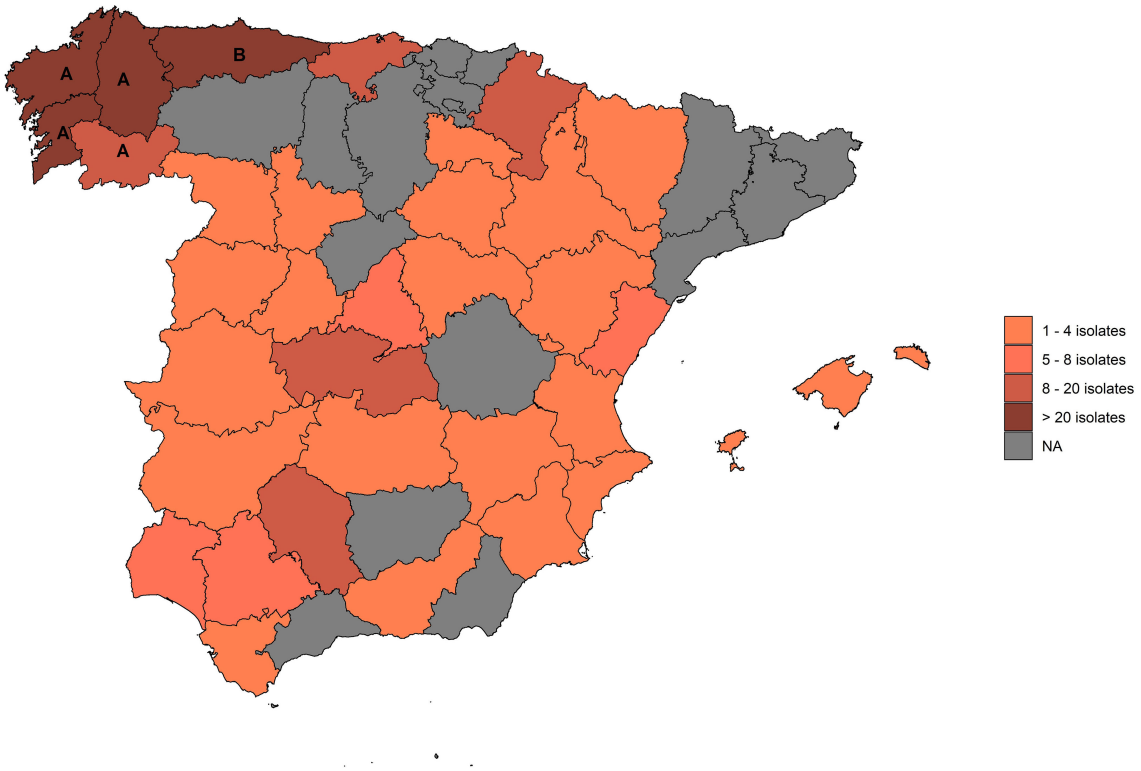

FIGURE 1
Provinces from which isolates included in this study originated. **(A)**: Galicia; **(B)**: Asturias.

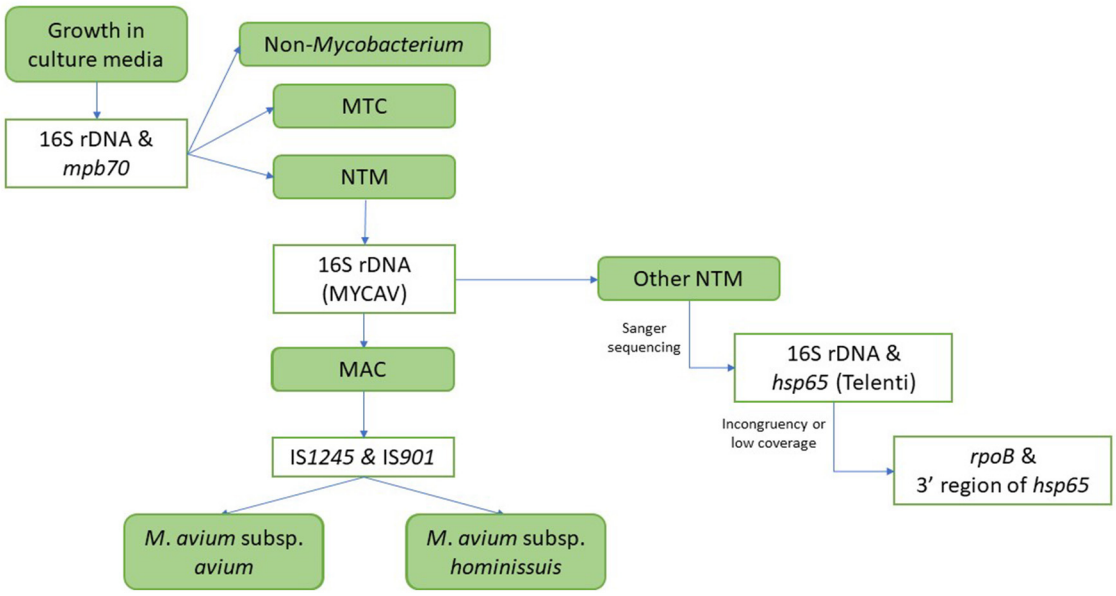

FIGURE 2
Workflow for the identification of isolates. MTC, *Mycobacterium tuberculosis* complex; NTM, non-tuberculous mycobacteria; MAC, *Mycobacterium avium* complex; MYCAV, MAC-specific 16S rDNA PCR.

NTM and 142 (46.1%) as MAC species. In the remaining 65 isolates (17.4% of all samples) a reliable identification was not achieved. Of them, 30 (46.2%) were classified as non-MAC NTM, 23 (35.4%) as MAC, and the 16S rDNA sequence revealed a bacteria not belonging to the *Mycobacterium* genus for the remaining 12 (18.5%) isolates.

Within the 166 non-MAC NTM isolates, 27 species were reliably identified (Table 1), being the most frequent species *M. nonchromogenicum* ($n = 85$; 51.2%), followed by *M. bourgelatii* ($n = 11$; 6.6%), *M. shimoidei* (8; 4.8%), *M. kansasii* (7; 4.2%) and *M. intermedium* (7; 4.2%). A reliable identification was not achieved in 30 isolates (15.3%), of which 8 and 16 did not yield a positive result for the *rpoB* and 3′ region of *hsp65* genes, respectively (Table 2). Among these isolates, we identified 23 "genotypes" present in 1–5 isolates. A genotype is defined as distinct partial 16S rDNA and *hsp65* (Telenti fragment), and, when available, *rpoB* and 3′ region of *hsp65* sequences. Most of the non-MAC NTM were retrieved from Galicia (141/196). However, the diversity of genotypes was almost the same as in the non-OTF regions (29 and 28 genotypes, respectively).

For the 142 MAC isolates five species were identified (*Mah*, *Maa*, *M. intracellulare*, *M. yongonense*, and *M. colombiense*). Of these, *M. avium* subspecies accounted for 92.3% ($n = 131$) of the retrieved isolates, with *Mah* (98; 69.0%) being more represented than *Maa* (33; 23.2%). Most MAC isolates (63/165) originated from Asturias since this region submitted primarily cultures preliminarily identified as MAC, but 56 and 46 MAC isolates from Galicia and non-OTF regions, respectively, were also included. Regarding the predominant MAC species, *Mah* was recovered more frequently than *Maa* in all regions, and in the case of Galicia, *M. intracellulare* (4) was identified even more frequently than *Maa* (1).

Of the 23 isolates with non-reliable identification, we did not obtain a PCR amplicon for the *rpoB* and 3′ region of *hsp65* genes in 11 and 20 isolates, respectively. Among these 23 isolates, seven genotypes, as previously defined, were identified in between 1 and 10 isolates each. The most common genotype was found in 10 isolates from five provinces in which a 99.8% similarity with the 16S rDNA sequence of *M. colombiense*, *M. intracellulare*, and *M. bouchedurhonense* coupled with a 97.4% similarity with the *hsp65 M. scrofulaceum* gene sequence was obtained. All genotypes for isolates with a non-reliable identification are shown in Table 2.

Finally, the 16S rDNA sequence revealed that 12 isolates did not belong to the *Mycobacterium* genus but instead to the following species: *Prauserella rugosa* ($n = 3$), *Streptomyces hydrogenans* (3), uncultured actinobacteria (3), *Corynebacterium pseudotuberculosis* (2), and *Brevibacillus brevis* (1).

Overall, macroscopical granulomatous bTB-like lesions were observed in lymph node samples from 20 animals (5.3%). From these samples, 11 different species were identified: *Mah* ($n = 6$), genotype 2 (2) (Table 2), *M. nonchromogenicum* (2), *M. shimoidei* (2), *Maa* (1), *M. intracellulare* (1), *M. xenopi* (1), *Corynebacterium pseudotuberculosis* (1), *M. interjectum* (1), *M. yongonense*, and genotype 7 (1) (Table 2).

TABLE 1 NTM species with a reliable identification from cattle positive to skin test.

NTM species	Isolates (%[a])
M. avium subsp. *hominissuis*	98 (31.8%)
M. nonchromogenicum	85 (27.6%)
M. avium subsp. *avium*	33 (10.7%)
M. bourgelatii	11 (3.6%)
M. shimoidei	8 (2.6%)
M. intracellulare	7 (2.3%)
M. kansasii	7 (2.3%)
M. intermedium	7 (2.3%)
M. alsense	5 (1.6%)
M. parascrofulaceum	5 (1.6%)
M. smegmatis	5 (1.6%)
M. xenopi	4 (1.3%)
M. abscessus	3 (10.7%)[b]
M. palustre	3
M. colombiense	2
M. engbaekii	2
M. fortuitum	2
M. gilvum	2
M. holsaticum	2
M. interjectum	2
M. thermoresistibile	2
M. triplex	2
M. yongonense	2
M. aubagnense	1
M. bohemicum	1
M. chitae	1
M. frederiksbergense	1
M. heckeshornense	1
M. heraklionense	1
M. koreense	1
M. lentiflavum	1
M. paraense	1

[a]Percent relative to the 308 isolates with identification.
[b]This represents the aggregated percentage of those species with less than four isolates.

4 Discussion

Non-tuberculous mycobacteria are one of the limiting factors compromising diagnostic performance of bTB tests (15, 32). As the burden of bTB is decreasing in most countries in which eradication campaigns have been consistently applied, the need to maintain a high specificity is key to keeping the positive predictive value as high as possible, but without compromising the overall sensitivity of the surveillance system. The characterization of the factors affecting diagnostic specificity, such as the most prevalent NTM associated with non-specific reactions to bTB skin tests, can be useful to design strategies aiming at minimizing their impact. Here, we present a thorough characterization of a large panel of NTM isolates retrieved from skin test reactor cattle from OTF herds to assess their diversity in Spain.

A great percentage of the isolates included in the study belonged to MAC (specifically to the *Mah*, *Maa*, *M. intracellulare*, *M. colombiense*, and *M. yongonense* species, of which the first two were

TABLE 2 NTM isolates without a reliable identification from cattle positive to skin test.

Genotype	Isolates	16S rDNA	% of similarity	*hsp65* (Telenti fragment)	% of similarity	*rpoB*	% of similarity	3′ region of *hsp65*	% of similarity
1	10	*M. colombiense/intracellulare/bouchedurhonense*	99.8	*M. scrofulaceum*	97.4	*Mycobacterium* sp.[a]	100	*M. timonense*	96.1
2	5	*M. marseillense/yongonense/intracellulare*	100	*M. avium/colombiense*	98.6	*M. mantenii*	96.2	–	–
3	5	*M. vaccae*	100	*M. vaccae*	93.4	–	–	–	–
4	2	*M. barrassiae/mengxianglii*	99.0	*Mycobacterium* sp.[b]	95.7	–	–	–	–
5	2	*M. chelonae/phlei*	99.8	*M. chelonae/phlei*	99.5	*M. chelonae/phlei*	99.6	–	–
6	2	*M. colombiense/intracellulare/bouchedurhonense*	100	*M. avium/colombiense*	98.5	*M. mantenii*	96.2	–	–
7	2	*M. colombiense/intracellulare/bouchedurhonense*	100	*M. avium/colombiense*	99.1	*M. vulneris*	99.9	–	–
8	2	*M. colombiense/intracellulare/bouchedurhonense*	100	*Mycobacterium* sp.[c]	99.8	–	–	–	–
9	2	*M. szulgai/angelicum*	99.7	*M. saskatchewanense/nebraskense*	95.9	*M. paraense*	92.0	*M. parmense*	94.2
10	1	*M. algericum/sinense/novum*	99.2	*M. algericum/terrae/sinense*	100	–	–	*M. novum*	97.5
11	1	*M. bacteremicum/neoaurum/sphagni*	100	–	–	–	–	–	–
12	1	*M. brasiliensis*	99.0	*M. komanii*	97.6	*Mycobacterium* sp.[d]	100	–	–
13	1	*M. colombiense/intracellulare/bouchedurhonense*	100	*Mycobacterium* sp.[e]	99.8	–	–	–	–
14	1	*M. engbaekii*	100	*M. arupense*	99.3	–	–	*M. virginiense*	96.8
15	1	*M. flavescens*	99.1	*M. monacense*	96.2	*M. baixiangningiae*	96.0	–	–
16	1	*M. interjectum/paraense*	100	*M. lentiflavum/genavense*	96.7	*M. interjectum/paraense*	97.6	–	–
17	1	*M. interjectum*	100	*M. saskatchewanense*	97.0	*M. interjectum*	98.8	–	–
18	1	*M. marseillense/yongonense/intracellulare*	100	*M. yongonense/intracellulare/avium*	99.8	–	–	–	–
19	1	*M. nonchromogenicum*	100	*M. icosiumassiliensis*	97.8	–	–	–	–
20	1	*M. novum/sinense/algericum*	100	*M. senuense*	100	–	–	*M. novum/sinense*	100
21	1	*M. scrofulaceum/paraffinicum*	100	*M. bohemicum*	96.6	*M. seoulense*	95.8	*Mycobacterium* sp.[f]	95.4

(Continued)

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TABLE 2 (Continued)

Genotype	Isolates	16S rDNA	% of similarity	*hsp65* (Telenti fragment)	% of similarity	*rpoB*	% of similarity	3′ region of *hsp65*	% of similarity
22	1	*M. septicum*	100	*Mycobacterium* sp.[g]	99.5	*Mycobacterium* sp.[h]	100	–	–
23	1	*M. terrae*	99.6	*M. parascrofulaceum*	100	*M. terrae*	94.6	–	–
24	1	*M. triplex*	99.0	*M. genavense*	97.2	–	–	*Mycobacterium* sp.[f]	95.4
25	1	*M. triplex*	99.7	*M. avium* subsp. *hominissuis**	98.8	–	–	–	–
26	1	*M. triplex*	99.7	*M. parmense*	98.0	*Mycobacterium* sp.[i]	97.9	–	–
27	1	*M. triplex*	99.7	*M. avium* subsp. *paratuberculosis/avium/hominissuis**	98.1	–	–	–	–
28	1	*M. vanbaalenii*	100	–	–	*M. aurum*	94.4	–	–
29	1	*M. vanbaalenii/vaccae*	99.7	–	–	*M. vaccae*	96.8	–	–
30	1	*Mycobacterium* sp.[j]	99.9	–	–	*M. heraklionense*	90.6	–	–

[a]Sequence ID: ON994929.
[b]Sequence ID: HF566126.
[c]Sequence ID: OK539021.
[d]Sequence ID: KM234049.
[e]Sequence ID: EU619890.
[f]Sequence ID: CP079863.
[g]Sequence ID: KT4550001.
[h]Sequence ID: OK538910.
[i]Sequence ID: ON994981.
[j]Sequence ID: MK890524.
*These samples were negative to MAC-specific 16S rDNA PCR (MYCAV).

10.3389/fvets.2024.1361788

by far the most common), partly due to the fact that the second region from which a higher number of isolates were available (Asturias) provided mostly NTM preliminary identified as MAC (Table 1) (33). Nevertheless, the high frequency of isolation of MAC members from cattle here is in agreement with previous studies in which certain MAC members (*Mah*, *Maa*, *Map*, *M. arosiense*, *M. bouchedurhonense*, *M. chimaera*, *M. colombiense*, *M. intracellulare*, and *M. vulneris*) were also isolated from bovine samples and identified as a potential cause of non-specific reactions (15, 19, 34). The potential to elicit a cross-reacting immune response in bTB diagnostic tests by MAC members (and to some extent also by other NTMs) should be reduced by the use of the CIT, which also considers the response elicited by the avian protein purified derivative (PPD-A) obtained from a *Maa* isolate (35, 36), so diagnostic interference should be limited in areas where this test is used (33). This, however, can lead to a decrease in diagnostic sensitivity of between 14 and 44 percentage points according to some estimates (37), and therefore comparative tests should be used when the risk of tuberculosis is considered low. In our study, most of the animals (353/373) were reactors in the SIT (based only on the bovine PPD), and in fact, of the only 20 animals that were reactors in the CIT (all coming from a current OTF area), *M. nonchromogenicum* was isolated from 13 of them and only one MAC species (*M. colombiense*) was isolated, suggesting that the CIT could be compromised to a larger extent by certain mycobacterial species.

M. nonchromogenicum was the most frequently isolated non-MAC NTM. This species was the most common NTM retrieved in samples from cattle in Northern Ireland (23 of 48 animals) (18) and Hungary (30 of 104) (17), and the second most common in France (81 of 310) (15), although in these studies not all samples came from skin-positive animals or OTF herds. *M. nonchromogenicum* has also been described in cattle samples with lesions collected at abattoirs in Switzerland (6), South Africa (34) and Ethiopia (7, 38), as well as in milk samples from positive cows to the CIT in Brazil (39) and in the nasal mucosa of cattle (40). In another study carried out in Spain (19), including tissue samples from cattle and wild boar in the Basque country (from which no samples were included here), *M. nonchromogenicum* was isolated in three of 21 SIT positive cattle and nine wild boars. These results, coupled with our findings, demonstrate that *M. nonchromogenicum* can be found in reactor cattle from Northern regions in Spain, from which most isolates were retrieved, and could be a relevant contributor to the development of cross-reactions in the skin test. The ability of *M. nonchromogenicum* to elicit reactions after the inoculation of certain mycobacterial antigens (including PPD-A and PPD-B) was explored in an experimental infection model in guinea pigs, but the observed induced reactions were limited (16). Similarly, the ability of this species to cause false positive reactions in skin tests in cattle has not been demonstrated yet, since experimental studies to assess the cross-reaction potential of NTM have been only limited to *M. kansasii* (4, 41) and *M. fortuitum* (42).

Regarding the geographical distribution of non-MAC NTMs, 93% of the 85 *M. nonchromogenicum* isolates identified here were recovered from cattle from Galicia, representing 56% of all non-MAC NTMs available from this particular region. In contrast, no non-MAC NTM accounted for more than 10% of the isolates retrieved from non-OTF regions, suggesting a more heterogeneous distribution of NTM species. When considering MAC isolates, *Mah* was the most commonly found species, but its relative frequency compared with other MAC species varied between regions: in Galicia 85.4% of all MAC isolates with a reliable identification (41/48) were *Mah* and only one *Maa* isolate was retrieved. In contrast in Asturias 34 *Mah* and 25 *Maa* isolates were identified, accounting for 57.6 and 42.4% of the 59 MAC isolates with reliable identification, respectively, while in non-OTF regions *Mah* and *Maa* represented 65.7 and 20.0% of the 46 MAC isolates available from these regions, respectively. Although these differences may be pointing out at differences in the epidemiology of cross-reactions due to NTM in cattle between OTF and vs. non-OTF regions, the highly heterogeneous sampling efforts may have led to biases contributing to these results and thus these findings should be interpreted with care. Differences in the most prevalent NTM species associated with cross-reactions were not influenced by the production type, with *M. nonchromogenicum* being the most abundant in both beef and dairy herds in Galicia (42.3 and 38.4% of all isolates in each type, respectively). Similarly, the distribution of MAC members across different production type herds in Asturias was also similar, with *Mah* being isolated in 52.5 and 56.5% of the beef and dairy herds, respectively, and *Maa* in 40.0 and 34.8% of them. This suggests that the prevalence of the NTM may be more influenced by regional factors rather than linked to the type of production.

Interestingly, 53 isolates in our study could not be reliably assigned to a specific species. Among them, 10 isolates coming from beef herds exhibited identical sequences in the four sequenced genes (genotype 1, Table 2), which would constitute the fifth most common "species" in our study. These isolates had a similarity of 99.8% with *M. colombiense/intracellulare/bouchedurhonense* (MAC members) based on the 16S rDNA and of 100% with a sequence previously classified as belonging to MAC based on the *rpoB* gene (19). However, the closest match to their *hsp65* short sequence was *M. scrofulaceum* (97.4% of similarity), while the highest similarity with a MAC member *hsp65* sequence was only 95.7%, below the 97.3% threshold previously suggested for MAC members for this gene (33). This result highlights the importance of using multiple genetic markers to achieve a comprehensive identification of the species involved (28, 43). Further studies would be required to conclude on the nature of these isolates.

Our results demonstrate that multiple NTM species could be associated with the occurrence of non-specific reactions in the skin test in cattle in Spain. Nevertheless, the relative importance of MAC members (especially *Mah* and *Maa*) and *M. nonchromogenicum*, which constituted up to 69% of all isolates considered in the study, demonstrates that certain species are more likely to lead to diagnostic interferences in OTF herds in low prevalence/OTF areas of the country, in agreement with what has been described in other European (6, 15, 17–19) and non-European countries (34, 38, 39).

By including only isolates retrieved from positive animals in OTF herds (that maintained this status for at least 3 years after the reactor was found) we attempted to minimize the chance that animals could have also been infected with a MTC member. Further studies based on guinea pig and cattle experimental models will be conducted to confirm the ability of the characterized strains

to induce non-specific reactions in non-bTB infected cattle, and to explore the ability of different antigens to discriminate the immune response induced in skin test and interferon-gamma assays. Altogether, this will contribute to limit the interference in the results of routine diagnostic tests, which can compromise the reliability of the herd status obtained and the non-desirable risk management measures that are necessary to implement in the context of an eradication programs.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, OR642807—OR642833, OR648408—OR648468, OR671981—OR672046.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because no need. All the animals were slaughtered due to positive results to the official skin test following the Bovine Tuberculosis Eradication Program in Spain.

Author contributions

AG-B: Data curation, Formal analysis, Writing—original draft, Writing—review & editing. JAl: Data curation, Funding acquisition, Resources, Supervision, Writing—review & editing. JB: Funding acquisition, Writing—review & editing. JM: Conceptualization, Supervision, Writing—review & editing. JAm: Data curation, Resources, Writing—review & editing. JS: Investigation, Resources, Writing—review & editing. LdJ: Data curation, Funding acquisition, Writing—review & editing. BR: Data curation, Methodology, Supervision, Writing—review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. AG-B holds a PhD fellowship (reference CT58/21-CT59/21) from the Universidad Complutense de Madrid and Banco Santander. This work was funded by the European Union Reference Laboratory for Bovine Tuberculosis, the Spanish Ministry of Agriculture, Fisheries and Food, and is a contribution to the project Integrated Strategies for Tuberculosis Control and Eradication in Spain (ERATUB) (RTI2018-096010-BC22, Ministerio de Ciencia, Innovación y Universidades, MICINN).

Acknowledgments

The authors would like to thank the collaboration and support of the EU-RL for Bovine Tuberculosis, the Spanish Ministry of Agriculture, Fisheries and Food, the Consellería do Medio Rural of the Xunta of Galicia, and to the Consejería de Medio Rural y Política Agraria of the Principado de Asturias. Also, we would like to thank Francisco Lozano, Nuria Moya, Tatiana Alende, Alexandra Gutiérrez, Cristina Viñolo, Daniela de la Cruz, and Laura Sánchez Martín from the Mycobacteria Unit of VISAVET Health Surveillance Center for their technical support.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1361788/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 01 February 2024
DOI 10.3389/fvets.2024.1334036

OPEN ACCESS

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RECEIVED 06 November 2023
ACCEPTED 11 January 2024
PUBLISHED 01 February 2024

CITATION
Di Marco Lo Presti V, Ippolito D, Migliore S, Tolone M, Mignacca SA, Marino AMF, Amato B, Calogero R, Vitale M, Vicari D, Ciarello FP and Fiasconaro M (2024) Large-scale serological survey on *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep and goat herds in Sicily, Southern Italy. *Front. Vet. Sci.* 11:1334036. doi: 10.3389/fvets.2024.1334036

Large-scale serological survey on *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep and goat herds in Sicily, Southern Italy

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Introduction: Paratuberculosis (PTB) is a worldwide chronic, contagious enteric disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) mainly affecting ruminant species. PTB is a WOAH-listed disease with direct and indirect economic losses in the livestock sector, negative impact on animal welfare and significant public health concerns. In spite of this, MAP prevalence in small ruminants is still unknown and the prevalence appears to be underestimated in many countries. The aim of this study is providing a first large-scale serological survey on MAP infection in small ruminants in Sicily, a region of Southern Italy with the 11.3 and 8.9% Italian national heritage of sheep and goats, respectively.

Methods: For this purpose, we analyzed a total of 48,643 animals reared in 439 flocks throughout Sicily. MAP seroprevalence was estimated both at herd-level and animal-level within breeds reared in all the nine sampled provinces.

Results: Our results revealed a high overall apparent prevalence at herd-level of 71.8% in sheep and 60.8% in goat farms with an animal-level prevalence of 4.5 and 5.1% in sheep and goats, respectively. Significant statistical differences were found between the provinces and within the breeds both in sheep and goats.

Discussion: Our study provides the first large-scale serological survey on PTB infection in small ruminants in Sicily and showed a high prevalence of disease depending to the species, breed and province. This study represents the first step to better understand the MAP epidemiology in a typical Mediterranean breeding context, suggesting the need of in-depth study on the herds risk factors, including the eventual presence of candidate genes for resistance/susceptibility to PTB in native breeds.

KEYWORDS

paratuberculosis, seroprevalence, sheep, goat, *Mycobacterium avium* subsp. *paratuberculosis*, Italy, Sicily

1 Introduction

Paratuberculosis (PTB) or Johne's disease is a chronic, contagious infectious disease that affects the enteric tract of domestic and wild ruminants. The causative agent of PTB is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which is mainly transmitted by the fecal-oral route and then spreads horizontally and vertically (1). The disease has been known and studied for over two centuries and it still represents a significant concern, both for the serious zoo-economic losses in infected herds and for its zoonotic potential (2).

Despite the lack of certainty on the cause/effect relationship, MAP is believed to be implicated in the pathogenesis of Crohn's disease in humans, in consideration of the high probability of isolation of MAP in affected patients (3, 4). Furthermore, it is suspected that MAP may also be implicated in the pathogenesis of other diseases such as type 1 diabetes mellitus (5), multiple sclerosis (6), Parkinson's disease (7), Blau syndrome (8), Hashimoto's thyroiditis (9), and in other autoimmune diseases, although no firm evidence has been scientifically confirmed.

The main routes of infection of MAP in humans are represented by environmental contamination, drinking water (10) and the food chain (11). Milk and dairy products have been implicated in the animal-to-human transmission, both if consumed raw or pasteurized (12). Indeed, MAP is characterized by a high resistance in the environment and can survive the standard commercial pasteurization (11).

The confirmation of MAP as a zoonotic agent would make mandatory the implementation of control and eradication procedures, which would inevitably lead to major issues in terms of public and veterinary health and severe economic losses for the entire livestock sector.

Given the sanitary and economic relevance of PTB, information on the occurrence and prevalence of MAP are available from many countries but limited to cattle dairy herds, in which control plans are also in place. In contrast, few studies in small ruminants have been published worldwide and consequently, prevention and control programs have not been established in many countries. Both seroprevalence and risk factors study are needed, especially in semi-extensive and extensive breeding contexts, in order to plan adequate strategies for the control and eradication of disease. In ruminants, PTB is clinically evident during the later stage of the infection. Symptoms such as significant weight loss, emaciation, spontaneous death may be only apparent in advanced stages (13). Subclinically infected animals can eliminate MAP through faeces even during the early phase of the infection, making difficult the control of the disease (13, 14). Goats appear to be more susceptible than sheep and both species are likely to develop the clinical signs of the disease (15).

PTB is globally widespread. It has been reported in several countries, such as Italy (16), Germany (17) and France (18), as well as in Asia, Africa and Oceania (19, 20). Caprine PTB was reported in Canada (21), USA (22) and Brazil (23). In New Zealand, the disease is endemic and widespread in sheep and dairy goats (24). In the Middle East and Africa, PTB was reported in sheep and goats in Saudi Arabia (25), Jordan (26), Egypt (27), Sudan (28), Morocco (29) and South Africa (19).

Italy is one of the largest European countries in terms of number of sheep and goat herds with an estimated population of around 5,9 million sheep and around 1 million goats (Ministero della Salute, Sistema Informativo Veterinario – Statistiche. https://www.vetinfo.it/j6_statistiche/#/report-pbi/89, accessed on June 30, 2023) and Sicily is the second Italian region for the number of sheep (662,305 heads, 11.3% of the national heritage) and third, after Sardinia and Calabria, for goats (90,926 heads, 8.9% of the national heritage). Sheep and goat breeding in Sicily is mainly semi-extensive, characterized by pastures, sometimes shared with other flocks, and a supplementary diet especially in the dry seasons. The island's biodiversity is enriched by a large number of autochthonous breeds (4 sheep and 5 goats) which constitute an invaluable heritage as a source of high-quality milk requested for typical dairy products (30). Genetic susceptibility to MAP infections in several small ruminant breeds has been investigated using quantitative and/or molecular genetics and despite low hereditability, all studies confirm genetic influence on paratuberculosis susceptibility (31). However, further genomic explorative studies to identify candidate genes and evaluate their prevalence are needed in small ruminants, especially in native breeds.

The aim of our study is to conduct the first large-scale investigation of MAP seroprevalence in small ruminants throughout Sicily providing the basis for further studies on risk factors analysis and genetic susceptibility in native breeds.

2 Materials and methods

2.1 Ethical statement

This study did not involve controls under EU Directive 2010 (2010/63/EU) and blood collection was not required with the benefit of animal welfare. The large-scale study on MAP seroprevalence in small ruminant herds in Sicily was carried out on sera samples collected by official regional veterinary services during the annual brucellosis monitoring plans in force in Sicily.

2.2 Study area

The study was carried out throughout the regional territory of Sicily, Southern-Italy, the largest island in the Mediterranean. It covers an area of 25,707 km2, including minor islands, and it is divided into nine provinces: Palermo, Trapani and Agrigento in the west, Caltanissetta and Enna in the center and Ragusa, Siracusa, Catania and Messina in the east (Figure 1).

2.3 Study animals

The analyzed samples belonged to dairy sheep and goats raised mainly in a semi-extensive system. Serum samples were obtained from 48,643 small ruminants, comprising 35,663 sheep and 12,980 goats. Regarding the sheep, we analyzed samples from crossbreed (18,848) and three different Sicilian native breeds: Comisana (3,914), Pinzirita (2,560) and Valle del Belìce (8,210). In goats we analyzed samples from crossbreed (7,746) and five different Sicilian native breeds: Derivata di Siria (453), Girgentana (324), Maltese (645), Messinese (1,088) and Argentata dell'Etna (7,746).

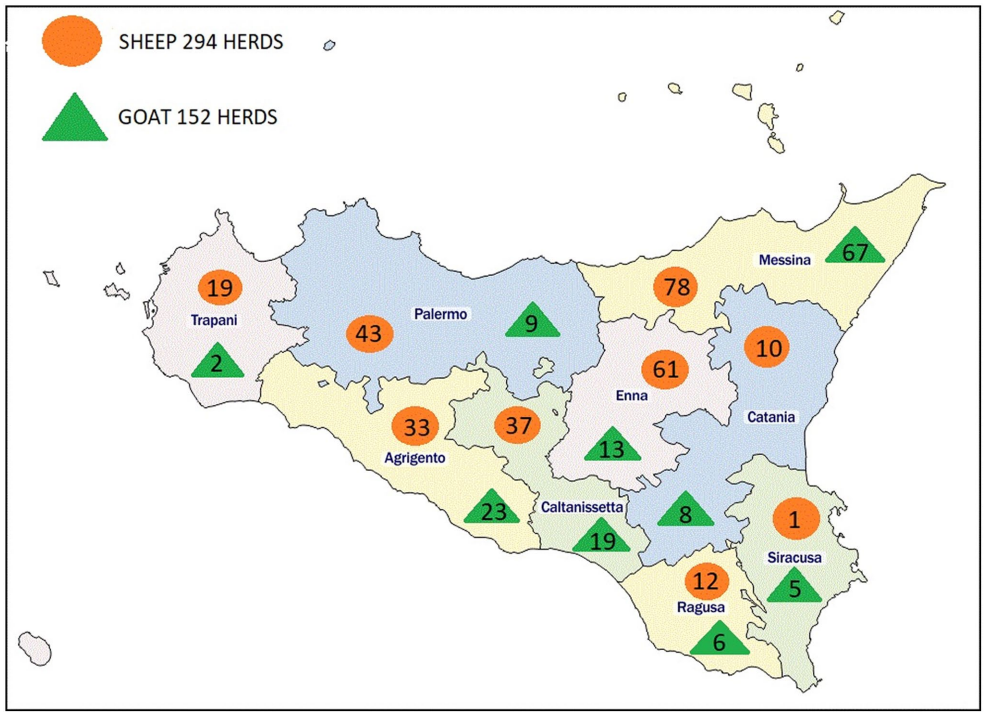

FIGURE 1
Map of the study area with sampled sheep and goat farms for province.

2.4 Study design and sampling method

Based on a previous study on the MAP seroprevalence carried out in sheep and goat in Sicily (Guercio et al., personal communication), we considered an estimated prevalence of 18% at herd level. Regarding the estimated prevalence at the animal level (32), reported a prevalence of 2%, obtained by Agar Gel Immunodiffusion Assay (AGID) method. However, given the low sensitivity in the preclinical phase of AGID method to detect MAP antibodies (26.8%) (33), the estimated prevalence at animal level was set at 5%.

Herds were selected by simple random sampling from official flock registers of Regional Government of Sicily. Vaccination plans against MAP have never been carried out in all sampled herds.

2.5 Sample size determination

The minimum sample size was calculated considering the total number of herds in Sicily (8,504 ovine herds and 3,121 caprine herds), using WinEpi software (http://www.winepi.net/, accessed on February 6, 2023) with 5% of precision and 95% confidence level (CI). Sampling was stratified by province based on the proportion of sheep and goats in each province. The stratified sampling was applied to calculate the minimum sample size at animal level, considering the number of heads reared in each farm by dividing the herds in six size class (from <50 to >1,000 animals). The total number of sheep or goat for each size class herds was calculated using WinEpi software (http://www.winepi.net/, accessed on February 6, 2023) with 5% of precision and 95% CI. In addition, seroprevalence was calculated within sheep and goat breeds at herd and animal level limited to the herds in which this information was available: 290 sheep herds (34,825 animals) and 149 goat herds (12,033 animals).

2.6 Serological analysis

Following serological testing for brucellosis, sera were collected, archived and stored at −18°C awaiting the analyses by the serology laboratory of Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri," during the 3 years in study. All samples were examined by IDEXX Paratuberculosis Screening Ab Test [IDEXX (IDEXX Laboratories, Inc., Westbrook, ME, United States)] according to the manufacturer's instructions. Positive samples were confirmed using IDEXX paratuberculosis verification Ab test (IDEXX Laboratories, Inc., Westbrook, ME, United States), following manufacturer's instructions.

For calculating the true prevalence (TP), we used the Sensitivity (Se) and Specifity (Sp) data indicated by the producer: Se = 34.9% and Sp = 97.3% in sheep and Se = 51% and Sp = 94.8% in goats. Considering the low sensitivity of the test, we calculated the TP according to Se and Sp indicated in a review of 2009 by Nielsen & Toft (Se = 37% Sp = 98.5% in sheep and Se = 73% and Sp 97.5% in goats).

The TP was calculated using the Epitools calculation system which applies the corrective formula of Rogan-Gladen (34).

Since both Se and Sp at herd level are a function of sample size (n), and as the sample sizes per herd in this study varied considerably, the TP was estimated only at animal-level whereas apparent prevalence (AP) was used also at herd-level.

2.7 Statistical analysis

As previously mentioned, the Epitools software was utilized to calculate the AP and TP. The same software was employed to determine the confidence intervals for both AP and TP estimations, consistent with Brown et al. (35), along with the positive predictive value (PPV) and negative predictive value (NPV). The chi-square test was used to assess provincial and breed-within-species differences in apparent prevalence using the chi-square function within the R software version 4.2.2 ($p < 0.05$).

3 Results

3.1 Seroprevalence at the herd level vs. individual level

In the face of 439 herds to be sampled (227 and 212 sheep and goat respectively), a total of 446 herds (294 and 152 sheep and goat respectively) in nine provinces were finally included in this study (Table 1).

The minimum sample size for both sheep and goat herds was achieved in six provinces: Agrigento, Caltanissetta, Enna, Messina, Trapani and Ragusa. In the remaining three provinces (Palermo, Siracusa, and Catania), the minimum sample size was not achieved, with sample coverage ranging from 10 to 78.2%.

The overall mean of the regional AP in sheep herds was 71.8% (95% CI: 53.2–90.4%) (Table 2) with a highest value (100%) in Ragusa (12 herds) and Siracusa (just one sampled herd) provinces, while lower prevalence was found in Trapani (19 herds) and Messina (78 herds) with AP of 26.3 and 46.2%, respectively. Statistical differences between the provinces were found ($p < 0.001$).

The overall mean of the AP in goat herds was 60.8% (95% CI: 37.5–84.1%) (Table 3) with a highest prevalence of reported in Siracusa (100%; 5 herds), Caltanissetta (84.2%; 19 herds), Ragusa (83.3%; 6 herds) and Catania (75%; 8 herds) provinces. In contrast the lowest AP was found in Agrigento province (39.1%, 23 herds). Statistical difference between the provinces was found ($p < 0.05$).

At animal-level we found 1,577 positive sheep with an overall mean of the AP of 4.5% (95% CI: 2.1–6.8%). The TP were 6.8% (95% CI: 0.8–12.9%) and 8.6% (95% CI: 2.3–14.9%) according to IDEEX and Nielsen & Toft, respectively (Table 2). The highest AP (9.4%) was found in Catania province (TP reported was 20.8 and 22.2% according to IDEXX and Nielsen & Toft values, respectively). The lowest values were found in Trapani and Siracusa provinces, with AP of 0.8 and 1.4%, respectively (Table 2). The PPV and NPV were 0.42 and 0.96, respectively. Statistical differences between the provinces were found ($p < 0.001$).

The overall mean of the AP at animal-level resulted 5.1% (95% CI: 3.4–6.8%) in 12,980 goats with TP of 8.2 (95% CI: 3.9–12.6%) and 10.2% (95% CI: 5.3–15.0%) according IDEXX and Nielsen & Toft values, respectively. The highest AP was found in Enna provinces (7.8%; 657 animals) with a resulted higher TP of 15.8 and 17.7% according to IDEEX and Nielsen & Toft values, respectively. Lowest AP were found in Siracusa (602 animals) and Messina (7,325 animals) with 1.5 and 1.7%, respectively. TP resulted nearly 0% in both cases. The PPV and NPV were 0.32 and 0.99, respectively.

TABLE 1 Proportion of sheep and goat herds for province.

Province	% of sheep herds	% of goat herds
Agrigento	11.2	7.5
Caltanissetta	5.7	6.5
Catania	6.9	6.6
Enna	13.5	1.2
Messina	19	38
Palermo	24.5	25.8
Ragusa	7.3	2.1
Siracusa	4.3	7.0
Trapani	7.4	4.0
	100	100

3.2 Seroprevalence within breeds

Regarding the prevalence study within the sheep breeds, at herd-level the AP ranged from 47.9% in Pinzirita (2,560 animals) to 93.7% in crossbreed (18,848 animals), while at animal-level the AP ranged from 2.7% in Pinzirita to 5.7% in Comisana (3,914 animals) (Table 4). The TP at animal-level showed the higher values in Comisana (9.3 and 11.8% according to IDEEX and Nielsen & Toft, respectively) and Valle del Belice (6.5 and 9.3% according to IDEEX and Nielsen & Toft values, respectively). Lowest values of the TP were found in Pinzirita (0 and 3.4% according to IDEEX and Nielsen & Toft, respectively) and crossbreed (2.2 and 5.3% according to IDEEX and Nielsen & Toft, respectively). Worthy of mention, the highest AP of 18.7% found in Sarda breed (995 animals; 2 herds), with TP values slightly less than 50% according both IDEEX and Nielsen & Toft (data no show in table). Regarding the AP at herd-level within the goat breeds, we found lower overall seroprevalence than sheep breeds, with values between of 16.7 and 67.7% in Girgentana (324 animals) and Messinese (1,088 animals), respectively. Conversely, the AP at animal-level within goat breeds showed a wider range of data than sheep breeds, ranged from 0.6% in Girgentana to 8.8% in Argentata dell'Etna (1,777 heads). The TP obtained was very high in Argentata dell'Etna according both IDEEX (18.9%) and Nielsen & Toft (20.5%) values. High TP values was also found in Maltese (9.9 and 12.3% according to IDEEX and Nielsen & Toft values respectively). Conversely, TP of 0% was found in Girgentana and Derivata di Siria (453 animals) (Table 5).

4 Discussion

Our study provides the first large-scale overview on MAP infection in small ruminant throughout Sicily, estimating the herd-level, animal-level and within breeds seroprevalence. Our investigation revealed a high herd-level AP of 71.8% in sheep and 60.8% in goat herds with values ranking from 26.3 to 100% and 39.1 to 84.2% depending on the province, in sheep and goat herds, respectively.

Regarding the animal-level prevalence, we found a regional AP of 4.5% in sheep with the highest values of 9.4% reported in Catania province. Overall AP within-herd in goats was 5.1%, highest value of

TABLE 2 Overall apparent (AP) and True prevalence (TP) of MAP at herd and animal level in sheep, considered by provinces.

Species	Province	Sampled farms	AP herd-level %	Sampled heads	AP animal-level %	TP IDEXX Se = 34.9% Sp = 97.3%	TP Nielsen & Toft Se = 37% Sp = 98.5%
Sheep	Agrigento	33	75.8 (58.98–87.17)	4,356	6.6 (5.89–7.36)	12.1 (9.9–14.5)	14.4 (12.4–16.5)
	Caltanissetta	37	83.8 (68.86–92.35)	4,424	7.6 (6.85–8.40)	15.2 (12.9–17.7)	17.2 (15.07–19.50)
	Catania	10	80.0 (49.02–94.33)	1,324	9.4 (7.91–11.05)	20.8 (16.19–25.95)	22.2 (18.06–26.91)
	Enna	61	73.8 (61.56–83.16)	7,180	3.9 (3.48–4.37)	3.7 (2.41–5.19)	6.8 (5.57–8.09)
	Messina	78	46.2 (35.53–57.14)	9,883	2.3 (2.02–2.60)	0.0	2.2 (1.46–3.13)
	Palermo	43	60.5 (45.58–73.63)	2,915	2.4 (1.91–3.02)	0.0	2.5 (1.14–4.29)
	Ragusa	12	100.0 (75.75–1.00)	3,764	5.9 (5.19–6.70)	9.9 (7.73–12.41)	12.4 (10.39–14.64)
	Trapani	19	26.3 (11.81–48.79)	1,539	0.8 (0.45–1.36)	0.0	0.0
	Siracusa	1	100	278	1.4 (0.56–3.64)	0.0	0.0
		294	**71.8 95% CI (53.24–90.40%)**	**34,086**	**4.5 95% CI (2.1–6.8%)**	**6.8 95% CI (0.8–12.9%)**	**8.6 95% CI (2.3–14.9%)**

Significance ($p \leq 0.05$) of χ^2 test between provinces.

7.8% in Enna province was found. According to IDEEX and Nielsen & Toft values respectively, TP ranged from 6.8 to 8.6% in sheep (Table 2) and between 8.2 and 10.2 in goats (Table 3).

To date, preliminary studies have already investigated the presence of MAP infection in sheep herds in Sicily, reporting a seroprevalence of 18% in Palermo (Guercio et al., personal communication) and 3.4% in Trapani (36) provinces. Our results show higher prevalence in both provinces (60.5 and 26.3% in Palermo and Trapani respectively) proving that MAP infection was previously underestimated in these provinces and probably in the rest of the region. In Italy, although there are no large-scale surveys on the spread of MAP infection in small ruminants, a significative study carried out in Apulia (a region sited in Southern Italy) revealed a herd-level AP of 60.5%, with 3% at sheep-level and 14.5% at goat-level (16). Another study performed on dairy sheep in Marche region (central Italy) showed a higher prevalence of 73.7% at flock-level with 6.29% within-herd (37).

Concerning the MAP infection in goats, our results overlap other studies carried out recently in four Northern Italy concerning 33 dairy herds, reporting a seroprevalence of 58% at herd-level with 7.4% at animal level (38).

Regarding other livestock species, a serological study in dairy cows carried out in two neighboring Northern Italian regions (Lombardy and Veneto) that account for over 50% of the Italian dairy cattle population reported a herd-level apparent prevalence of 48 and 65%, respectively (39). Similar results was obtained in a large-scale survey in water buffaloes (*Bubalus bubalis*) in Campania reporting an apparent prevalence of infection of 54.7% (40). Our results support previous studies and confirm the high prevalence of MAP infection in Italian livestock.

Concerning the global epidemiological situation of MAP infection in small ruminants, the number of prevalence studies is low and as they differ in study design and diagnostic tests used. Consequentially, MAP prevalence in small ruminants is still unknown in many countries and the prevalence appears to be underestimated (41). According to a global survey involving 48 countries, limited to countries with available data, the estimated herd-level prevalence of MAP was higher than 10% in 5 of 11 countries for sheep and 7 of 12 countries for goats (42). The same study reported estimated seroprevalence data at animal level up to 5% in four countries both in sheep and goats and values higher of 15% in two and three countries in sheep and goats, respectively (42). Finally, Seroprevalence of MAP by ELISA in sheep and goats in different European countries was summarized by Jiménez-Martín et al. recently (43).

Regarding the hypothesis that some small ruminant breeds are more resistant to MAP infection than others, experimental data are very limited and evidence of experimental infection on different breeds is lacking. Several studies report that some sheep breeds may develop clinical signs of PTB rather than others (44–46). Other studies showed that genetics may play a role in the susceptibility to PTB in sheep and goats (31, 47). Within the small ruminant breeds tested in study, we found AP values at animal-level between 2.7 (Pinzirita) and 5.7% (Comisana) in sheep and 0.6% (Girgentana) and 8.8% (Argentata dell'Etna) in goats. Notable of TP differences between the breeds stand out, with values between 0 (Pinzirita) and 11.8% (Comisana) in sheep and 0 (Derivata di Siria and Girgentana) and 20.5% (Argentata dell'Etna), suggesting the hypotetical implication the of genetic factors in the predisposition to MAP infection. In addition, the high seroprevalence values at animal-level (AP 18.7%; TP ~50%) in the two Sarda herds included in the study do not allow an analysis of statistical significance, but suggest the need for further investigations on this breed that appears to be the most bred in Italy.

The breeding of native breeds in Sicily represents an important source of income for the livestock sector especially to produce typical dairy products derived from raw milk which support people in rural where a semi-extensive farming is in place and PTB is rarely diagnosed.

Considered a similar breeding system that exposes all animals to several risk factors for PTB infection in Sicily, our findings strengthen the hypothesis that genetic factors within breed may determine susceptibility/resistance to MAP infection and highlight the importance of the preservation of native breeds as a reservoir of

10.3389/fvets.2024.1334036

TABLE 3 Overall apparent (AP) and True prevalence (TP) of MAP at herd and animal level in goats, considered by provinces.

Species	Province	Sampled farms	AP herd-level %	Sampled heads	AP animal-level %	TP IDEXX Se = 34.9% Sp = 97.3%	TP Nielsen & Toft Se = 37% Sp = 98.5%
Goat	Agrigento	23	39.1 (22.16–59.21)	819	6.6 (5.09–8.50)	12.1 (7.42–18.03)	14.4 (10.11–19.73)
	Caltanissetta	19	84.2 (62.43–94.48)	1,768	5.8 (4.78–6.96)	9.6 (6.44–13.21)	12.1 (9.23–15.37)
	Catania	8	75.0 (40.93–92.85)	493	6.5 (4.64–9.02)	11.8 (6.01–19.63)	14.1 (8.83–21.18)
	Enna	13	69.2 (42.37–87.32)	657	7.8 (5.95–10.06)	15.8 (10.10–22.87)	17.7 (12.54–24.12)
	Messina	67	52.2 (40.49–63.75)	7,325	1.7 (1.42–2.01)	0	0.5 (−0.22–1.45)
	Palermo	9	44.4 (18.88–73.33)	348	4.3 (2.63–6.99)	5.0 (0–13.32)	7.9 (3.18–15.46)
	Ragusa	6	83.3 (43.65–96.99)	948	6.8 (5.32–8.53)	12.7 (8.14–18.10)	14.9 (10.77–19.80)
	Trapani	2	0.0	20	5.0 (0.89–23.61)	7.1 (−7.59–64.95)	9.86 (−3.5–62.29)
	Siracusa	5	100.0 (56.55–1.00)	602	1.5 (0.79–2.82)	0	0
		152	**60.8 95% CI (37.5–84.1%)**	**12,980**	**5.1 95% CI (3.4–6.8%)**	**8.2 95% CI (3.9–12.6%)**	**10.2 95% CI (5.3–15.0%)**

Significance ($p \leq 0.05$) of χ^2 test between provinces.

TABLE 4 Overall apparent (AP) and True prevalence (TP) of MAP at herds and animal level in sheep, considered by breeds.

Species	Breeds	Sampled farms	AP herd-level	Sampled heads	AP animal-level	TP IDEXX Se = 34.9% Sp = 97.3%	TP Nielsen & Toft Se = 37% Sp = 98.5%
Sheep	Comisana	41	53.7 (38.75–67.94)	3,914	5.7 (5.01–6.47)	9.3 (7.19–11.7)	11.8 (9.9–13.99)
	Pinzirita	48	47.9 (34.47–61.67)	2,560	2.7 (2.14–3.40)	0	3.4 (1.79–5.34)
	Valle Del Belice	106	50.0 (40.65–59.35)	8,201	4.8 (4.36–5.29)	6.5 (5.16–8.04)	9.3 (8.06–10.67)
	Crossbreed	93	93.5 (86.63–97.01)	18.848	3.4 (3.15–3.67)	2.2 (1.40–3.01)	5.3 (4.65–6.11)

Significance ($p \leq 0.05$) of χ^2 test between sheep breeds.

natural resistance against some infectious diseases. In this regard, genetic resistance/susceptibility to infectious diseases, in particular to Scrapie and Maedi-Visna virus (48–50) in Sicilian small ruminant breeds were already reported.

According to specific criteria of the European Union Animal Health Law – Regulation (UE) 2016/429 (AHL), PTB was included under Category E (listed disease for which there is a need for surveillance within the Union, of which Article 9(1)(e) for the listed animal species Bison spp., Bos spp., Bubalus spp., Ovis spp., Capra spp., Camelidae and Cervidae. Inclusion in category E entails the obligation of surveillance as well as notification of the disease to the competent authorities. Other categories were disregarded, mainly due to the low individual sensitivity of diagnostic tests currently in use, together with the difficulties of declaring countries, areas and herds paratuberculosis-free officially (42). To date, MAP infection prevalence in ruminants is considered underestimated worldwide. In Italy, the Ministry of Health, in application of the AHL, recently has approved the adoption on the national territory of "*Guidelines for the surveillance, the adoption of control plans and the assignment of health qualification to establishments of sensitive species (Cattle, Buffalo, Sheep, Goats) against paratuberculosis*" (GU General Series n.10 of 13-01-2023). These guidelines originate from the need for surveillance of the PTB throughout the national territory, in view of recognized endemicity in Italian herds and the awareness of having data that underestimate both its prevalence and incidence, these, essential for effective surveillance; its application, has as general objective of providing the right indications to implement surveillance on the national territory (definition of the roles, tasks and responsibilities of public and private veterinarians and operators; definition of "suspected" and "confirmed" cases and on the rules of reporting and notification of PTB). Despite the profound socio-economic impact of PTB in small ruminants, few studies have been published worldwide and consequently prevention and control programs have not been established in many countries.

Unfortunately, large-scale studies on the seroprevalence of PTB still presuppose limitations that we have also encountered. The sample sizes per herd in our study varied considerably, thus the TP was estimated only at animal-level and not at herd-level. In addition, the low sensitivity of the available diagnostic tests hampers the precision of diagnosis in subclinical infection and/or in early stage of disease with consequent underreported outbreaks.

Our study represents the first large-scale survey on the prevalence of PTB in small ruminants in Sicily, according to the Italian Ministry of Health guidelines for surveillance of the disease throughout the

TABLE 5 Overall apparent (AP) and True prevalence (TP) of MAP at herd and animal level in goats, considered by breeds.

Species	Breeds	Sampled farms	AP herd-level	Sampled heads	AP animal-level	TP IDEXX Se = 34.9% Sp = 97.3%	TP Nielsen & Toft Se = 37% Sp = 98.5%
Goat	Derivata di Siria	32	65.6 (48.31–79.59)	453	0.9 (0.34–2.25)	0	0
	Girgentana	12	16.7 (4.7–44.8)	324	0.6 (0.17–2.22)	0	0
	Maltese	9	44.4 (18.88–73.33)	645	5.9 (4.32–7.98)	9.9 (5.04–16.41)	12.3 (7.95–18.26)
	Messinese	31	67.7 (50.14–81.43)	1,088	3.5 (2.56–4.76)	2.4 (−0.45–6.39)	5.6 (2.97–9.18)
	Argentata dell'Etna	5	40.0 (11.76–76.93)	1,777	8.8 (7.55–10.19)	18.9 (15.06–23.25)	20.5 (17.04–24.47)
	Crossbreed	63	61.9 (49.56–72.88)	7,746	1.9 (1.62–2.23)	0	1.1 (0.33–2.05)

Significance ($p \leq 0.05$) of χ^2 test between goats breeds.

national territory. The data obtained showed a high prevalence of the disease in Sicilian herds which varies according to the species, breed, and province, highlighting the need to implementing specific PTB control plans in small ruminants.

This study represents the first step to better understand the epidemiology of the disease in semi-extensive breeding contexts, typical of Mediterranean basin, suggesting the need for further investigations to evaluate all the risk factors in the herds, included the presence of candidate genes for resistance/susceptibility to PTB in native sheep and goat breeds to helping the implementation of control plans in the near future.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because this study did not involve controls under EU Directive 2010 (2010/63/EU) and blood collection was not required with the benefit of animal welfare. The large-scale study on MAP seroprevalence in small ruminant farms in Sicily was carried out on sera samples provided by the annual brucellosis monitoring plan in force in Sicily, collected and archived during the three years in study.

Author contributions

VD: Conceptualization, Formal analysis, Project administration, Supervision, Writing – review & editing. DI: Data curation, Writing – review & editing. SM: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. MT: Data curation, Formal analysis, Writing – review & editing. SM: Conceptualization, Investigation, Writing – review & editing. AMFM: Investigation, Writing – review & editing. BA: Investigation, Writing – review & editing. RC: Investigation, Writing – review & editing. MV: Formal analysis, Writing – review & editing. DV: Investigation, Writing – review & editing. FPC: Investigation, Writing – review & editing. MF: Conceptualization, Formal analysis, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Italian Ministry of Health [grant number RC IZSSI 12/16].

Acknowledgments

Authors would like to thank Dr. Giovanna Romeo, Dr. Carmelinda Biondo, and Dr. Chiara Calanni for their scientific and technical support.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 06 February 2024
DOI 10.3389/fvets.2024.1301667

OPEN ACCESS

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RECEIVED 25 September 2023
ACCEPTED 10 January 2024
PUBLISHED 06 February 2024

CITATION
Turco S, Russo S, Pietrucci D, Filippi A, Milanesi M, Luzzago C, Garbarino C, Palladini G, Chillemi G and Ricchi M (2024) High clonality of *Mycobacterium avium* subsp. *paratuberculosis* field isolates from red deer revealed by two different methodological approaches of comparative genomic analysis.
Front. Vet. Sci. 11:1301667.
doi: 10.3389/fvets.2024.1301667

High clonality of *Mycobacterium avium* subsp. *paratuberculosis* field isolates from red deer revealed by two different methodological approaches of comparative genomic analysis

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Mycobacterium avium subsp. *paratuberculosis* (MAP) is the aetiological agent of paratuberculosis (Johne's disease) in both domestic and wild ruminants. In the present study, using a whole-genome sequence (WGS) approach, we investigated the genetic diversity of 15 *Mycobacterium avium* field strains isolated in the last 10 years from red deer inhabiting the Stelvio National Park and affected by paratuberculosis. Combining *de novo* assembly and a reference-based method, followed by a pangenome analysis, we highlight a very close relationship among 13 MAP field isolates, suggesting that a single infecting event occurred in this population. Moreover, two isolates have been classified as *Mycobacterium avium* subsp. *hominissuis*, distinct from the other MAPs under comparison but close to each other. This is the first time that this subspecies has been found in Italy in samples without evident epidemiological correlations, having been isolated in two different locations of the Stelvio National Park and in different years. Our study highlights the importance of a multidisciplinary approach incorporating molecular epidemiology and ecology into traditional infectious disease knowledge in order to investigate the nature of infectious disease in wildlife populations.

KEYWORDS

***Mycobacterium avium* subsp. *paratuberculosis*, red deer, wild animals, Italy, whole genome sequencing, epidemiology**

1 Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP), a member of the *Mycobacterium avium* complex, is an important pathogen responsible for a chronic granulomatous enteritis known as paratuberculosis or Johne's disease (1). The genome of MAP shares very high homology with that of the other *Mycobacterium avium* subspecies, but, to date, some

sequences, such as the F57 sequence, have been found only in MAP isolates, and it is considered one of the more specific targets for the diagnosis of paratuberculosis by PCR (2). MAP has been isolated from both ruminant and non-ruminant hosts, and, among wild animals, the red deer (*Cervus elaphus*) is one of the more affected animal by this disease (3). For this reason, according to the new Animal Law Regulation 429/2016, by being a natural MAP reservoir, the red deer is a potential epidemiological risk for MAP transmission in the surrounding area. From a clinical perspective, the manifestations of paratuberculosis in red deer closely resemble those observed in cows: persistent diarrhoea, weight loss, and deteriorating body condition as the disease advances (4). For a genetic perspective, three major MAP strain types were initially identified according to the host species and based on restriction endonuclease analysis, DNA hybridisation, and pulsed-field gel electrophoresis (PFGE): (*i*) Type I or S type for sheep; (*ii*) Type II or C type for cattle; and (*iii*) Type III or "intermediate" between Type I and Type II (5–8). A fourth strain type named "Bison" or Type B was later isolated from bison (*Bison bison*) in Montana, United States and distinct from an Indian Bison Type (9, 10).

Numerous studies have already been addressed at catching the diversity among MAP field isolates from red deer in European Countries. In 2009, Stevenson et al. characterised 164 MAP strains isolated from 19 diverse host species through restriction fragment length polymorphism (RFLP), PFGE, amplified fragment length polymorphism (AFLP), and mycobacterial interspersed repeat unit-variable number tandem repeat (MIRU-VNTR) analyses. Their conclusions confirm the presence of the abovementioned strain types but clearly state that multiple genotyping techniques targeting different genetic markers are necessary to better discriminate against the homogeneous MAP population. This statement was further confirmed by Fritsch et al. (11), when reporting a directed epidemiological connection between wild red deer and farmed cattle. Only through a combination of two common SSR profiles, nine MIRU-VNTR patterns and nine IS900-RFLP patterns, it was possible to identify 17 different genotypes (11). Thus, taking into account that different genotypes can coexist within herds or in different species within the same habitat (11, 12), an additional epidemiological transmission study identified 15 MAP genotypes across multiple hosts by MIRU-VNTR analysis (13).

The first genome-wide single-nucleotide polymorphism (SNP) analysis of 141 global MAP isolates provided a greater resolution than the previous genotyping methods and confirmed a restricted genetic diversity with a low substitution rate (14, 15). This study, together with the dropping costs of the whole-genome sequence (WGS) analysis, paved the way for further MAP epidemiological studies (16–20).

A study carried out at the Stelvio National Park, a large protected area in the North of Italy, investigated the prevalence of the paratuberculosis in the red deer population and the genotype of the associated MAP (21). All isolates recovered from this latest study were type II and shared the same VNTR/SSR loci profile, suggesting a possible clonal infection.

In the present study, to further investigate the genetic diversity of this bacterium within the Stelvio National Park, we analysed the genomes of 15 MAP field isolates collected in a time range of 10 years using two different whole-genome sequence (WGS) methodology, a *de novo* assembly, and a reference-based approach. Furthermore, a comparative genomics analysis including MAP isolates from across the globe was carried out. Due to the low mutational rate (i.e., highly conserved genome) and in light of the disadvantages of the classical genotyping methodologies mentioned above, we believe that a combination of different next-generation sequencing (NGS) experimental approaches can benefit this field.

2 Materials and methods

2.1 Study area and sampling

The red deer study population analysed in this study inhabits the northwestern part of the Stelvio National Park, within the Province of Sondrio, central Italian Alps (46°28 0″N, 10°22′0″E). The population includes approximately 1,200 animals according to the annual counts, and their spatial distribution extends over 27,900 ha between 1,200 and 3,850 m a.s.l. (22). Year-around movements of individually marked deer and landscape features (ridges, valleys) support an absent or sporadic frequency of connection with other red deer populations (23). Sampling was carried out during the official culling plants within a temporal window of 10 years and was officially authorised by the "Istituto Superiore per la Protezione e la Ricerca Ambientale" (ISPRA) and the Italian Ministry of Environment (Prot. 48585/T-A25-Ispra).

Mycobacterium avium subsp. *paratuberculosis* field isolates herein analysed were recovered from faeces or intestines according to the procedure reported in the OIE Manual (24). Part of the isolates herein analysed have already been examined and resulted in all type II (type C) with the same VNTRs/SSRs profile (21), but in this case, we included all the isolates we were able to recover from our collection for a total of 15 isolates.

In addition to these field isolates, for phylogenetic and comparative genomics analysis, eight more MAP strains isolated from red deer worldwide were included in our study. According to their data availability, either raw reads (for the MAPMRI dataset) or complete genomes (for DT3 and K10 strains) were downloaded from the NCBI SRA database (Table 1).

2.2 Sequencing, *de novo* assembly, and annotation

Total DNA was extracted from a pure culture of 15 *Mycobacterium avium* isolates, according to the procedure described in Bolzoni et al. (17). The DNA was then sequenced independently with an Illumina NextSeq platform, producing paired-end reads of 150 bp. Raw read quality was evaluated with FastQC (25), and the reads were filtered using Trimmomatic v0.39 (26) with the following parameters: CROP:140 HEADCROP:25 SLIDINGWINDOW:4:25 AVGQUAL:25 MINLEN:36.

In parallel, the raw reads of the ERR0 dataset downloaded from the NCBI SRA database were subjected to quality check and reads trimming as well. Due to the shorter read length of this MAPMRI dataset (76 bp average), the trimming parameters were modified as follows: CROP:65 SLIDINGWINDOW:4:20 AVGQUAL:25 MINLEN:36.

The trimmed reads were then *de novo* assembled using SPAdes v3.15.4 (27) with default parameters and k-mers set to 21, 33, 55, and 77, with the exception of the MAPMRI dataset, where a k-mer length of 77 was not used. The assembly statistics were evaluated using QUAST 5.0 (28). Structural and functional annotations were performed with Prokka

10.3389/fvets.2024.1301667

TABLE 1 List of *Mycobacterium avium* field isolates used in this study.

Strain name	Isolation date	Host	Geographic origin	Accession number
M01	2016	*Cervus elaphus*	Italy	This study
M02	2009	*Cervus elaphus*	Italy	This study
M03	2016	*Cervus elaphus*	Italy	This study
M04	2013	*Cervus elaphus*	Italy	This study
M05	2020	*Cervus elaphus*	Italy	This study
M06	2020	*Cervus elaphus*	Italy	This study
M07	2020	*Cervus elaphus*	Italy	This study
M08	2020	*Cervus elaphus*	Italy	This study
M09	2020	*Cervus elaphus*	Italy	This study
M10	2019	*Cervus elaphus*	Italy	This study
M11	2019	*Cervus elaphus*	Italy	This study
M12	2013	*Cervus elaphus*	Italy	This study
M13	2013	*Cervus elaphus*	Italy	This study
M14	2012	*Cervus elaphus*	Italy	This study
M15	2012	*Cervus elaphus*	Italy	This study
MRI063	2005	*Cervus elaphus*	Germany	ERR037390
MRI064	2005	*Cervus elaphus*	Germany	ERR037391
MRI083	NA	*Cervus elaphus*	The Netherlands	ERR037959
MRI086	NA	*Cervus elaphus*	Czech Republic	ERR037962
MRI090	NA	*Cervus elaphus*	Argentina	ERR037966
MRI160	NA	*Cervus elaphus*	New Zealand	ERR248986
DT3	NA	*Cervus elaphus*	Great Britain	GCA_000240445.2
K10	NA	*Bos taurus*	United States	GCF_000007865.1

v1.14.5 (29), using the pre-built Prokka database, further supported by the MAP K10 annotation as the Reference genome. For both DT3 and K10 strains, already assembled, all of the above steps were skipped.

2.3 Pangenome and comparative analysis

For whole-genome comparison, we adjusted the pipeline used in Turco et al. (30). Briefly, all the genomes listed in Table 1 were reordered with MAUVE (31) towards K10 set as the reference genome and then aligned using the *progressiveMAUVE* algorithm with default parameters (31). The average nucleotide identity (ANI) was calculated on the entire genomes with MUMMER v3.1 within the pyANI script (32). Prokka results in a Gff3 format were given as input to Roary v1.7.7 (33) in order to get a core-genome alignment used to construct a maximum 133 likelihood (ML) phylogenetic tree with RAxML-HPC, setting the GTRCATI algorithm as the substitution model and 1,000 bootstraps (34).

The tree was visualised with FigTree v.1.4.4[1] and edited with Inkscape v0.92.[2]

A pangenome analysis was performed with Anvi'o v.7 (35), providing Prokka results in a GenBank format as input files, using NCBI BLASTP for amino acid sequence similarity search, the default minbit heuristic set to 0.5, and the MCL inflation parameter set to 10. Besides Prokka, all the genomes were further annotated using NCBI's Clusters of Orthologous Groups (COG) database (36) and Pfam (37). Bins for single-copy core gene (SCG), accessory, and singletons were retrieved and further analysed.

2.4 Identification of effector genes

Mycobacterium avium subsp. *paratuberculosis* effector genes were downloaded from the virulence factors of pathogenic bacteria (VFDB, http://www.mgc.ac.cn/VFs/main.htm, Accessed on March 1, 2023) and blasted on the MAP assemblies under comparison using NCBI BLASTn. Using an *in-house* python script, each effector was considered present if it showed a minimum of 70% of identity and at least 70% of query coverage. The results were then used to create a presence/absence binary matrix that was plotted as a heatmap of the presence/absence using the pheatmap package v1.0.12 (38) within the R environment v3.4.4.

2.5 Reference-based assembly

A reference-based assembly was also carried out to better evaluate the genetic diversity among the MAP isolates under comparison and, thus, to identify the possible genomic regions that did not map on the

1 http://tree.bio.ed.ac.uk/software/figtree

2 www.inkscape.org

reference genome and that are characteristic of these MAP isolates. For this purpose, the trimmed raw reads were aligned towards the K10 genome using BWA aligner v0.7.12 (39) with default parameters. Once obtained the alignment BAM files, the mapped and unmapped reads were retrieved with SAMtools v1.13 (40) and the SNP variants called with SAMtools and BCFtools (40), setting a minimum base quality of 50 and a minimum mapping quality of 30, as suggested by Bryant et al. (14). These SNPs variants were used to build an ML phylogenetic tree using RAxML-HPC, with GTRCATI algorithm as the substitution model and 1,000 bootstraps (34) and to extract the final consensus sequence from each isolate. The ANI of these consensus sequences was calculated with MUMMER v3.1 within the pyANI script (32). Moreover, as mentioned above, the unmapped reads were further *de novo* assembled with SPAdes v3.15.4 (27) and annotated with Prokka v1.14.5 (29). CDS were further inspected to identify possible deer-specific features and other bacteria origins, mapping them against the online NCBI database. All the calculations have been carried out at Cineca in the framework of the ELIXIR-IT HPC@CINECA program (41) and on the Tuscia-DIBAF HPC center.

3 Results

3.1 DNA sequencing and reads trimming

For the *in-house* sequenced samples, total DNA was extracted from each sample, as already reported (17) in the protocol and sequenced with an Illumina NextSeq platform using a paired-end sequencing method, while the MAPMRI dataset was downloaded from the SRA NCBI database (Table 1). For both datasets, the raw reads number, before and after trimming, together with their length, are shown in Supplementary Table S1. The stringency of the trimming parameters was chosen according to the FastQC results, and the quality of reads was re-checked after trimming. The trimmed raw reads were used for two distinct pipelines: a *de novo* assembly-based and a reference-based assembly method.

3.2 *De novo* assembly and features annotation

Trimmed reads assembled with SPAdes v3.15.4 (27) yielded a total number of contigs ranging from 461 (M07) to 2,374 (M15), with a total genome length spanning from 4,601,483 (M05) to 5,031,920 (M01) (Supplementary Table S2). The assembly QUAST statistics, shown in Supplementary Table S3, indicate an average GC content above 69% and the presence of several undetermined bases. Prokka annotation identified 4,734 CDS on average, with three rRNA, one tmRNA in all the samples, and ranging from 55 to 58 tRNAs (Supplementary Table S2). Furthermore, among the identified CDS, an average of 1,237 were shown to have hypothetical functions and 3,428 were instead codified for a feature related to K10 annotation.

3.3 Genome alignment and average nucleotide identity

For a whole-genome comparison to identify possible rearrangement and insertions/deletions (Indels) events, all the contigs were reordered towards the K10 genome and further aligned as shown in Supplementary Figure S1. The coloured blocks represent conserved genomic regions aligned to K10, either in forward (above the centre line) or reverse (below the centre line) orientation. Despite the overall generic conservation, there are blocks with a lower similarity profile that are coloured in white within the blocks. Completely missing coloured blocks indicate a deletion in that region, while regions outside the blocks may represent sequence elements specific to a particular genome, like in the cases of M01 and M04 (Supplementary Figure S1).

The colour gradient of the ANI heatmap indicates an ANI spanning from 98.8 up to 100% (Figure 1). The majority of the Italian field isolates are quite similar to each other, clustering together with the rest of the foreigner isolates and reference strains K10 and DT3 (Figure 1). However, two field isolates (M01 and M04) were clustered on their own with the lowest percentage of 98.8 with the reference K10 genome (see below). All the other isolates are grouped together, except for MRI086 from the Czech Republic and MRI083 from the Netherlands clustered apart, with an average of identity of 99.7%.

3.4 Pangenome analysis

The pangenome analysis identified a total of 107,458 genes arranged in 6,964 gene clusters (Figure 2). Among those, 870 gene clusters are defined as "core genome," shared between all the MAP isolates, and represent 37% of the total genome. The core genome was then used to build the phylogenetic tree shown in Figure 3. In line with the results shown in Figure 1 for the whole-genome comparison, the core genome confirms the different genomic characteristics of the isolates M01 and M04, which cluster on a separate branch. These two isolates show decreasing similarity to MRI160 from New Zealand, DT3 from Great Britain, and M10 from Italy. All of the remaining isolates belong to a single branch that includes the samples from Germany, Argentina, Czech Republic, and the Netherlands, which are different only from K10 (Figure 3).

In the pangenome analysis (Figure 2), 2,827 core gene clusters were present in one copy per genome (SCG, Single Core Genome). These elevated numbers indicate high genomic similarity with a low evolutionary rate. Besides the 2,336 accessory gene clusters, 931 gene clusters were defined as singletons, peculiar for one single isolate (Supplementary Figure S2A). A COG functional category could not be assigned to 2,995 gene clusters, 161 from the core genome, 515 SCG, 808 singletons, and 1,511 accessory gene clusters (Supplementary Figure S2B). On the contrary, the majority of the gene clusters belonged to COG category "R" of general functional prediction only (typically, prediction of biochemical activity), category "Q" of secondary metabolites biosynthesis and category "I" of lipid metabolism, with the rest of the singletons spread among the different categories (Figure 4). Interestingly, the isolate with the highest number of singletons was M11, with 250 singletons, followed by M01, M05, and M15 with 157, 74, and 72 singletons, respectively (Supplementary Table S4). Despite most of the singletons being annotated as hypothetical proteins without COG category assignment, other singletons belonged to COG "I," "Q," "R," and "S" categories (Supplementary Figure S3). No COGs have been observed for category "W" (extracellular structures) and very few COGs for category "N" (cell motility). Among these, of particular relevance are the singletons annotated as K10 MCE family protein (COG category

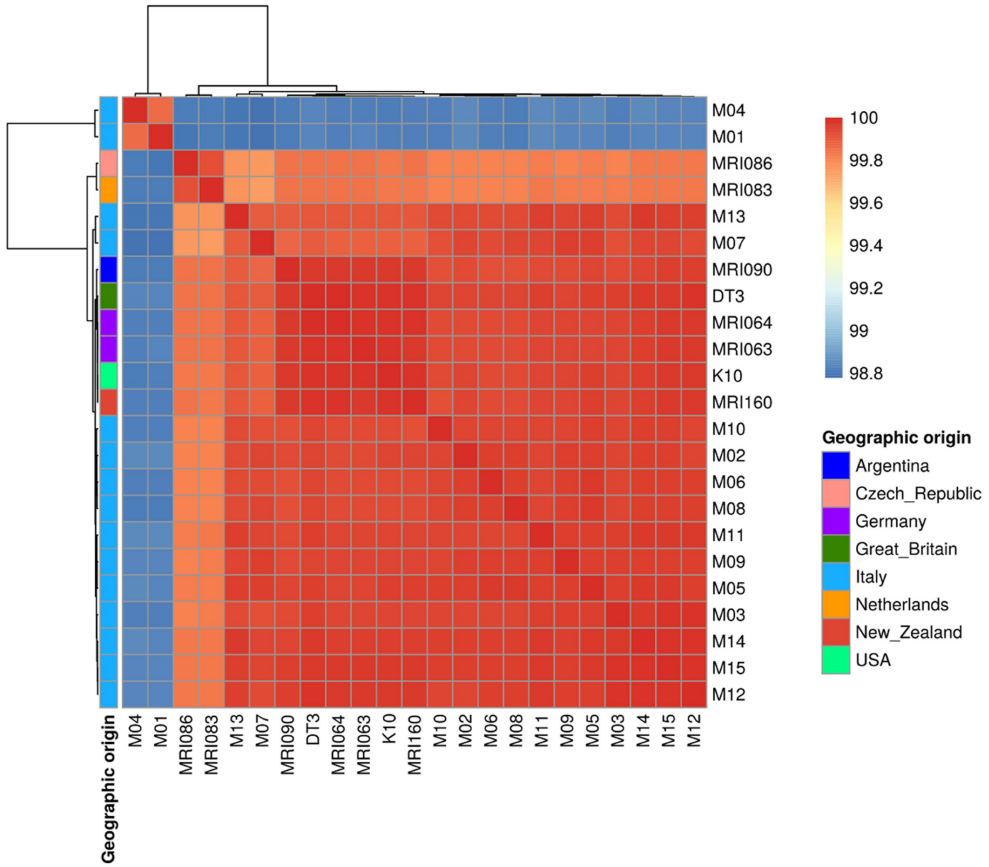

FIGURE 1
A Heatmap showing the Average Nucleotide Identity (ANI) among the MAP strains under comparison indicated as a percentage of identity ranging from 98.8 to 100%. The geographic origin of each sample is shown as well.

"M") and found in M01, M09, and M11, one singleton belonging to the K10 PPE family protein (COG category "S"), and both families associated with virulence, as well as transposase, DNA invertase that could contribute to this genome plasticity (Supplementary Table S4). Other singletons were annotated as TetR/AcrR transcriptional regulator family, besides several oxidoreductase, hydrolase, and recombinase. Overall, the Pfam annotations were comparable to those from COG and Prokka.

A relatively small number of genes (40) was present only in M01 and M04 samples, whose most frequent COG categories are "Energy production and conversion" and "Defence mechanisms" with eight and seven genes, respectively.

3.5 Virulence-related features

A total of 199 MAP virulence-related features (i.e., effector genes) from K10 were downloaded from the VFDB database and blasted on the MAP isolates under comparison. A total of 131 features were found in all the isolates, mostly related to immune modulation, nutritional or metabolic factors, effector delivery system, adherence, regulation, ABC transporters, and the MCE family proteins (Supplementary Table S5). Despite four effectors that were found only in K10 (effector delivery system RS21760, mycP2, mycP5, and PPE4), there is no particular virulence factor related only to the MAPs isolated from red deer (Figure 5). Interestingly, M01 and M04 are the only two isolates where psk2, ddrA, MAP_RS22660, and MAP_RS1926 were not present, confirming the peculiarity of these two isolates with respect to MAPs herein isolated.

3.6 Reference-based assembly

Independent to the *de novo* assembly pipeline described above, the trimmed reads were aligned to the reference K10 genome for a reference-based assembly using BWA with default parameters. After variant calling and SNP filtering according to base quality, mapping quality, and read support, a ML phylogenetic tree was built on the concatenated SNPs (Supplementary Figure S4). The high clonal level of the MAP isolates reconstructed with this method appears immediately clear, with all the samples clustering together, except for the M01 and M04 that in the *de novo* assembly already appeared different. To further verify this high genetic similarity, the consensus sequence was extracted from the aligned BAM file using SAMtools and BCFtools. The sequences were then aligned to each other and to the assembled K10 and DT3 to perform an ANI analysis, as shown in Supplementary Figure S5. Overall, the percentage of identity was higher than 99%, with the lowest similarity with M01 and M04, in line with the *de novo* assembly results previously presented. The F57 sequence was not found in these two last isolates.

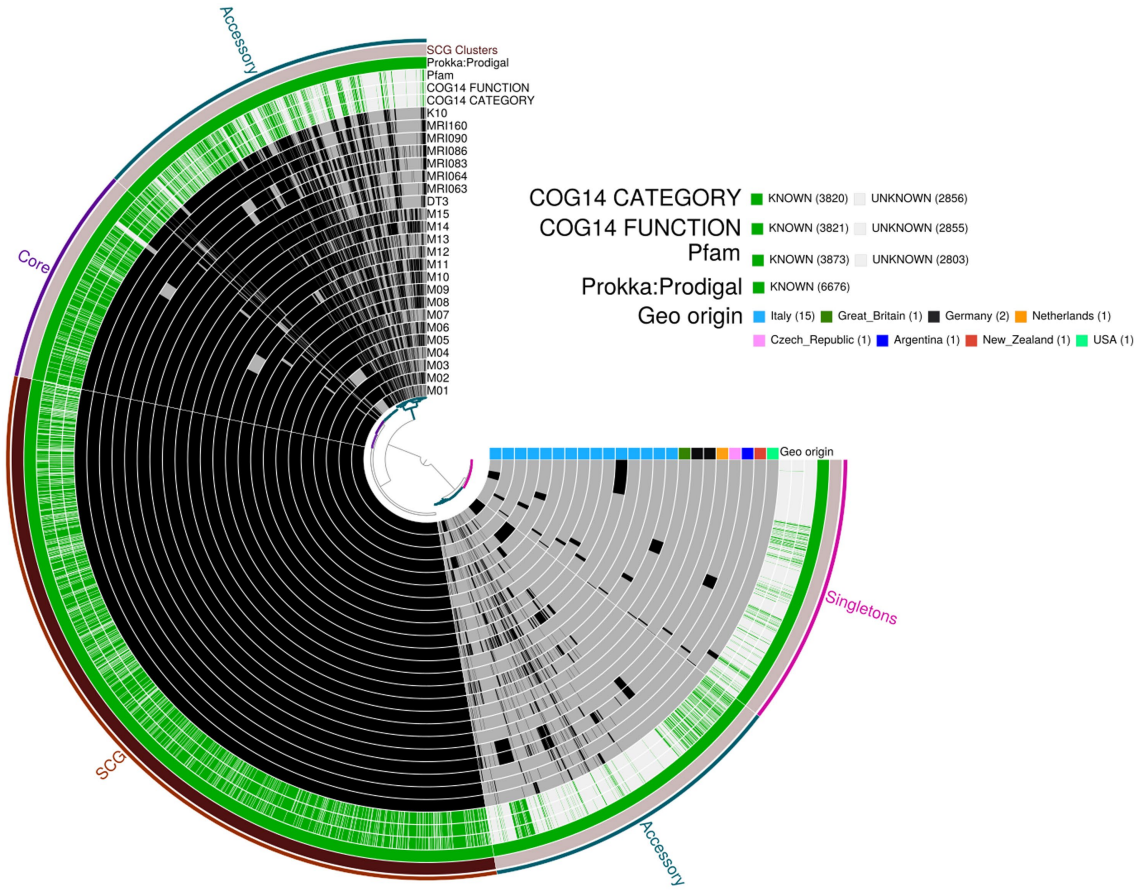

FIGURE 2
Pangenome analysis performed with Anvi'o. From the outermost to the innermost: (*i*) coloured bins indicating the accessory genes, the core genes, Single-Copy core Genes (SCG), and singletons; (*ii*) Single-Copy Gene cluster; (*iii*) Prokka: Prodigal, Pfam, COG functional annotation, and COG category annotation; and (*iv*) individual genomes organised regarding their phylogenetic relationships, with the dark colour indicating the presence of the gene cluster and the light colour its absence. Geographic origin and biovar affiliation are indicated as well.

Furthermore, the K10 unmapped reads from each sample were *de novo* assembled and annotated with Prokka database (Supplementary Table S6). Only two or three CDS (annotated mostly as hypothetical proteins) were identified, starting from less than 10 contigs for almost all the MAP isolates, while M01, M04, and M15 showed 580, 608, and 307 CDS, respectively. In line with previous results that showed the similarity between M01 and M04, 569 out of 580 CDS from M01 resulted to be 100% identical to the ones from M04. When blasted on the NCBI database, the major part of the common CDS (almost 60%) resulted similar to *M. avium* subsp. *hominissuis*, 28% to *M. avium* and only 0.44% to *M. avium* subsp. *paratuberculosis* (Supplementary Table S7). To confirm whether the two isolates belong to *M. avium* subsp. *hominissuis*, all the isolates were mapped against the *M. avium* subsp. *hominissuis* reference sequence (MA104—https://www.ncbi.nlm.nih.gov/datasets/taxonomy/243243/), following the same protocol used for K10 reference-based approach. The percentage of unmapped reads was 5.1 and 2% for M01 and M04, respectively; for the other isolates, the minimum was 7% for M06 and the maximum was 10.7% for M15 (Supplementary Table S6). Two of the remaining 11 CDS were related to K10 TauD/TfdA dioxygenase and HNH endonuclease (and indeed present as well in K10) and nine hypothetical proteins (SM1). When using BLAST from the NCBI database, the nucleotide sequence of these 11 CDS resulted more similar to *M. avium* subsp. *hominissuis*, besides few CDS related also to *M. avium* subsp. *paratuberculosis* and still with only hypothetical annotation. A total of 33 CDS from M04 were not present in both M01 and K10, even if 11 of them were annotated as spirocyclase, MMPL family transporter (considered a virulence factor in MAP) [Vilijoen et al., 2017 (42)], cytochrome P450, SDR family NAD (P)-dependent oxidoreductase, aldehyde dehydrogenase, twice TetR/AcrR, CbbQ/NirQ family protein, TNT antitoxin, and alpha/beta hydrolase related to K10 (SM2). When blasted, the nucleotide sequences of these 33 CDS appeared to be related to *M. conspicuum*, *M. intracellulare*, and *M. avium* subsp. *hominissuis*, confirming the peculiarity of sample M04. Plus, the M04 singletons identified by Anvi'o were also found among the unmapped CDS. None of the above CDS coming from both M01 and M04 were in common with M15. M15 singletons from Anvi'o analysis, instead, were found in the unmapped CDS as well, mostly related to transposase, oxidoreductase, heavy metal translocase, and teichoic acids export ATP-binding protein (Supplementary Table S4). Among the other dataset, MRI083 and MRI086 were the two with the highest number of unmapped contigs, codifying for 107 and 108 CDS, respectively (Supplementary Table S6).

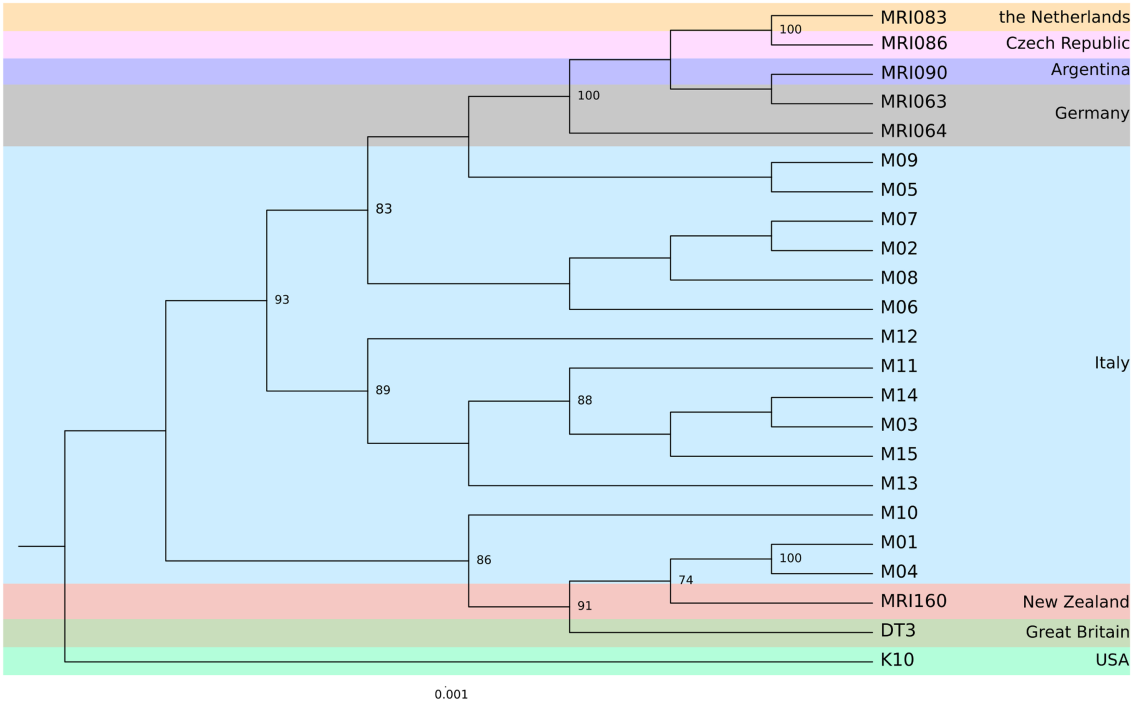

FIGURE 3
Maximum Likelihood (ML) phylogenetic tree based on the core genome alignment identified by Roary among the MAP strains from eight different geographic areas. The number of bootstraps above 70 is indicated as node labels.

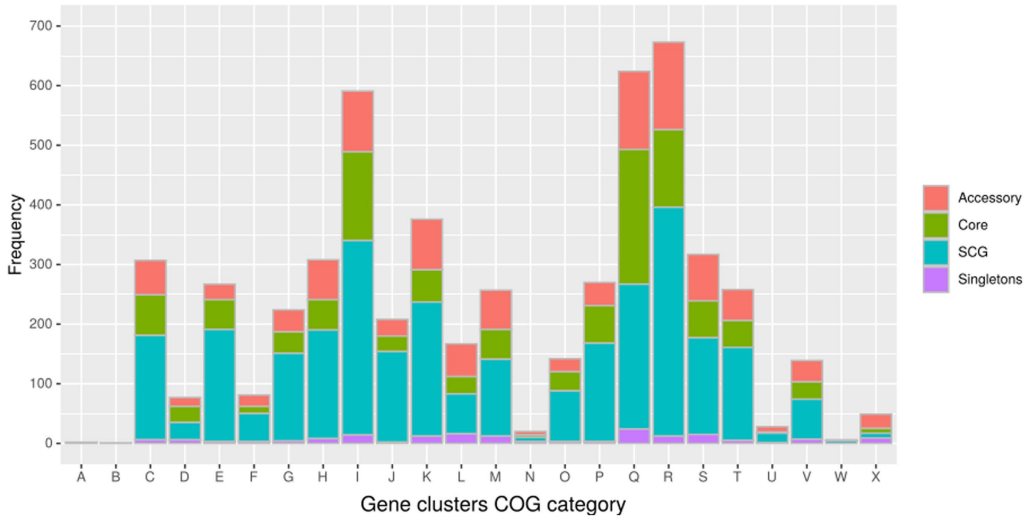

FIGURE 4
Distribution of the gene clusters identified by Anvi'o among the different Cluster of Orthologous groups (COG). **(A)** RNA processing and modification (not used for prokaryotic COGs); **(B)** chromatin structure and dynamics; **(C)** energy production and conversion; **(D)** cell cycle control and mitosis; **(E)** amino acid metabolism and transport: **(F)** nucleotide metabolism and transport; **(G)** carbohydrate metabolism and transport; **(H)** coenzyme metabolism; **(I)** lipid metabolism; **(J)** translation; **(K)** transcription; **(L)** replication and repair; **(M)** cell wall/membrane/envelope biogenesis; **(N)** cell motility; **(O)** post-translational modification, protein turnover, and chaperone functions; **(P)** inorganic ion transport and metabolism; **(Q)** secondary metabolites biosynthesis, transport, and catabolism; **(R)** general functional prediction only (typically, prediction of biochemical activity); **(S)** function unknown; **(T)** signal transduction; **(U)** intracellular trafficking and secretion; **(Y)** nuclear structure (not applicable to prokaryotic COGs); and **(Z)** cytoskeleton (not applicable to prokaryotic COGs).

4 Discussion

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a microorganism characterised by an extremely slow growth rate both in a controlled environment (*in vitro*) and within living organisms (*in vivo*). One of the consequences of this slow pace is a low mutation rate and the extremely well-conserved and closed core genome (14, 15, 17, 20). In the field of MAP epidemiology, traditional population genetic

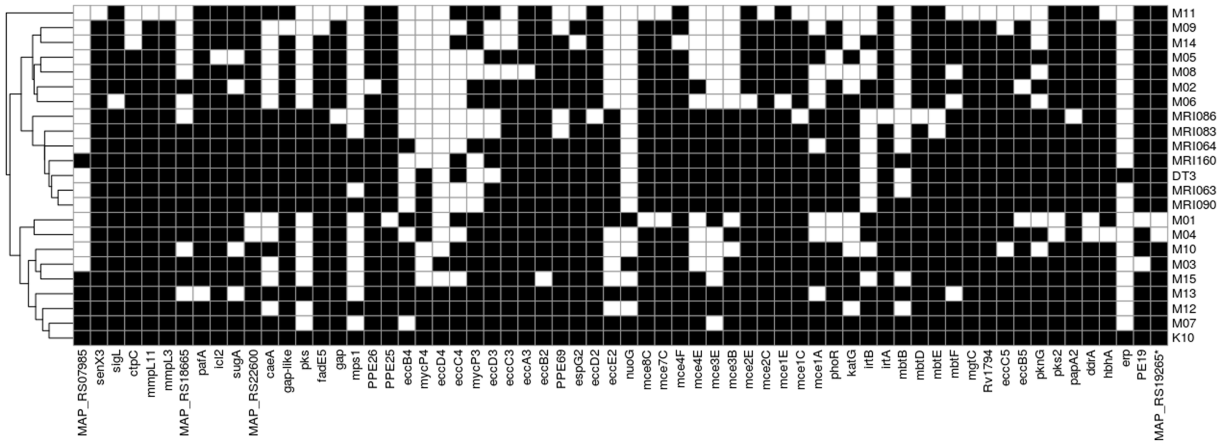

FIGURE 5
Distribution of K10 virulence-related features among the MAP strains. The black block indicates the presence of the feature retrieved by BLAST alignment, while the white block indicates its absence.

and diversity analysis methods such as VNTR or SSR analysis have currently been largely supplanted by WGS approaches. These new methods offer significantly higher discriminatory power, even in populations characterised by extremely low diversity, such as *Mycobacterium bovis* (43) and MAP (17, 44).

In this study, we have investigated the genomic structure of *Mycobacterium avium* isolated from red deer inhabiting the Stelvio National Park using WGS. Notably, a prior investigation focussing on a subset of the analysed field isolates had assessed the MAP population structure using minisatellite and microsatellite loci revealing only one distant profile (21), suggesting a clonal origin of infection.

Our WGS analysis, employing two different approaches—a *de novo* assembly and a reference-based reads alignment on MAP K10 isolate, largely confirms this initial observation, highlighting the presence of a single major clade. Moreover, our results unveiled the existence of other field isolates (M01 and M04) belonging to a different subspecies within the *Mycobacterium avium* species, the *Mycobacterium avium* subsp. *hominissuis*. Further analyses demonstrated a strong similarity between these two isolates markedly different in genome content from the other MAPs. Despite a substantial time gap between the isolation of these two field isolates, we hypothesise that there was no epidemiological link between the two infections. To reinforce this classification, both isolates lacked the F57 sequence, one of the most specific MAP markers (2). The presence of *Mycobacterium avium* subsp. *hominissuis* has previously been documented in red deer in Austria and Hungary (45, 46), Specifically, in Austria, instances were recorded during 2001–2002, while Rónai et al. (46) reported isolates collected between 2006 and 2015. This particular subspecies of *Mycobacterium avium* is considered an environmental bacterium, often found in water, soil, dust, or straw, and its primary hosts include pigs and humans (47).

The successful utilisation of both *de novo* assembly and reference genome alignment for exploring diversity within the MAP population has already been documented (18, 48). Notably, we believe that both approaches should be used to investigate the MAP population structure of field isolates apparently coming from the same outbreak or very close ones. While the *de novo* method circumvents biases related to indels or genetic translocations, the less computationally intensive reference genome alignment is appropriated for reconstructing and comparing core genomes. Consequently, building epidemiological trees based on SNP analyses of the core genome remains the predominant approach in evaluating potential epidemiological links within the MAP field, due to its notably low mutation rate (14, 17, 19, 20).

The analyses of the singletons of our red deer isolates revealed that the majority of COGs belong to "S" category (proteins with unknown function), followed by "I" category (lipid transport and metabolism), "Q" category (secondary metabolites biosynthesis, transport, and catabolism), "R" category (general function prediction only), and "K" category (transcription). In a recent study, Lim et al. (18), analysing different types of MAP (type I, type II, and type III), observed that the main part of COGs fell into the categories S (function only) and R (general function prediction only), and among the well-characterised genes, the major part mapped into categories was related to metabolism, in particular "Q," "I," and "K," eventually mirroring what we have observed in the present study for MAP type II. This observation suggests a possible common trend for all MAP isolates independent of the type analysed.

Focussing on the differences between the red deer isolates herein analysed and the K10 genome, it is worth noting that the absence in all our samples of RS21760, mycP2, mycP5, and PPE4. Myc P2 and P5 genes codify for two predicted serine proteases with unknown function (49), and the role of effector delivery system RS21760 is still unknown. The PPE proteins have conserved proline (P) and glutamic acid (E) residues in their N-terminal sequences and are involved in outer membrane nutrient transport and host–pathogen interaction or immune evasion (50). In more detail, PPE4 is important for mycobactin-mediated iron acquisition (50), and since it is absent in all isolates herein presented but K10, we could hypothesise that its presence in K10 is due to the adaptive stress for *in vitro* multiple passages.

We also observed differences in the two M01 and M04 isolates, which clustered differently with respect to the others. All the red deer samples, including K10, have in their genome the virulence-related features psk2, ddrA, MAP_RS22660, and MAP_RS1926 absent in M01 and M04. Moreover, the two field isolates M01 and M04 did not show in their genomes pks2, MAPRS 22660, MAPRS 19265, and ddrA. In more detail, ddrA gene codifies for a protein which is part of the ABC transporter complex DrrABC involved in doxorubicin resistance (51) and of the transport and synthesis of phthiocerol

dimycocerosate (PDIM) (52), a virulence factor of *Mycobacterium tuberculosis* (53); however, the pks2 gene codifies for a protein involved in sulpholipid biosynthesis (54). No information is currently available for the products of genes MAPRS 22660 and MAPRS 19265.

More importantly, in M01 and M04, the F57 sequence, one of the most specific MAP markers (2), is absent. This evidence is in line with the alignment results that assign M01 and M04 to *M. avium* susbsp. *hominissuis*.

5 Conclusion

The use of WGS in the field of MAP epidemiology confirms the clonal nature of the paratuberculosis outbreak present in the red deer inhabiting the Stelvio National Park, demonstrating the presence of a single major clade in a time range of a decade. We also identified the presence of two isolates belonging to *M. avium* subsp. *hominissuis* in the red deer population. Even if the reduced number of samples did not permit us to make any hypotheses about the origin of the infection and the evolution within the red deer population inhabiting the Stelvio National Park, it is worth noting these two isolates being isolated in two different locations of the Stelvio National Park and in different years.

In future investigations, it will be pivotal to acquire additional metadata for red deer at the individual and population levels and on MAP isolates from livestock sharing summer pasture to make hypotheses about the origin and the evolution of the multi-host infection. A multidisciplinary approach incorporating molecular epidemiology and ecology into traditional infectious disease knowledge will improve the prevention measures in a semi-extensive production system and support decision-making on a poorly addressed topic.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) are: https://www.ncbi.nlm.nih.gov/, PRJNA986832. The python script used for the analysis of the effector genes is available on request to daniele.pietrucci@unitus.it.

Ethics statement

This study uses strains obtained at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna. The Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna did not require the study to be reviewed or approved by an ethics committee because some field isolates have been obtained in the frame of Routine diagnostic activity at Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (faecal specimens). The others have been recovered from animals that have been culled for management purposes according to the official culling plan to reduce red deer density that has been authorised by Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), the Italian Ministry of Environment (Prot. 48585/T-A25-Ispra), in the Lombardy sector of the Park. Therefore, animals were not sacrificed for research purposes specific to this study.

Author contributions

ST: Investigation, Writing – original draft, Writing – review & editing. SR: Methodology, Writing – review & editing. DP: Methodology, Software, Writing – review & editing AF: Methodology, Writing – review & editing. MM: Writing – review & editing, Conceptualization, Investigation, Writing – original draft. CL: Conceptualization, Writing – review & editing. CG: Writing – review & editing, Funding acquisition, Resources. GP: Writing – review & editing, Methodology. GC: Conceptualization, Data curation, Investigation, Software, Writing – original draft, Writing – review & editing. MR: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported partially by the public health service grant no. E54I1900280001 (internal code PRC 2019/010) from the Italian Ministry of Health.

Acknowledgments

We thank Erika Scaltriti from IZSLER for the DNA sequencing with Illumina system. We also thank all the staff of the Stelvio National Park for their fundamental support in field activities and logistics. We acknowledge computing resources by Elixir-IT, Cineca and HPC-DIBAF.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1301667/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 14 February 2024
DOI 10.3389/fvets.2024.1346514

OPEN ACCESS

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RECEIVED 29 November 2023
ACCEPTED 29 January 2024
PUBLISHED 14 February 2024

CITATION
Barandiaran S, Ponce L, Piras I, Rosas AC, Peña Martinez J and Marfil MJ (2024) Detection of non-tuberculous mycobacteria in native wildlife species at conservation risk of Argentina. *Front. Vet. Sci.* 11:1346514. doi: 10.3389/fvets.2024.1346514

Detection of non-tuberculous mycobacteria in native wildlife species at conservation risk of Argentina

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Introduction: Non-tuberculous Mycobacteria (NTM) are mainly environmental but can cause opportunistic infections and diseases in humans and animals. Livestock and wild animals can be infected with NTM. In Argentina, there are native wild species facing conservation risks, and they are the focus of protection and reintroduction projects designed to preserve biodiversity in various ecoregions. The aim of this study was to report the presence of NTM in samples collected from four endangered native wild species from nine Argentine provinces, as part of their pre-release health assessment.

Methods: A total of 165 samples from giant anteater, peccary, tapir and pampas deer were obtained, these included either bronchoalveolar or endotracheal lavages, or oropharyngeal, nasopharyngeal or tracheal swabs. Bacteriological culture followed by molecular identification and sequencing were performed.

Results: A total of 27 NTM were detected, including *Mycobacterium avium* subsp. *hominissuis, M. intracellulare, M. terrae, M. gordonense, M. kumamotonense, M. fortuitum, M. saskatchewanense*, and *M. genavense*. Results revealed a 16,36% NTM recovery rate, with the giant anteater showing the highest prevalence among the mammals under study.

Discussion: In Argentina, due to extensive production systems, the interaction between domestic and wild species sharing the same environment is frequent, increasing the exposure of all the species to these NTM. In this way, the transmission of infectious agents from one to another is feasible. Moreover, NTMs might interfere with the diagnosis of bovine tuberculosis and paratuberculosis. These findings emphasize the importance of active health surveillance in conservation programs. It highlights the need to address NTM epidemiology in wildlife and its impact on conservation and public health.

KEYWORDS

non-tuberculous mycobacteria, native wildlife, conservation, bacteriological diagnosis, molecular identification

1 Introduction

The term "non-tuberculous mycobacteria" (NTM) is the most commonly used expression to refer to species of the genus *Mycobacterium* other than *Mycobacterium tuberculosis* (MTB) and *Mycobacterium leprae* (1). NTM encompasses saprophytic and opportunistic mycobacteria. Within this group, there is the *Mycobacterium avium*

-*intracellulare* complex (MAC), which includes mycobacteria that cause disease in various animal species, while behaving as an opportunistic pathogen in others (2). While mycobacteria within the MTB complex (MTBC) are primarily associated with clinical signs, the role of NTM causing diseases, mainly related with immunocompromised individuals, is increasingly being reported in both humans and animals (3–5).

Wild mammals are susceptible to pathogenic mycobacteria such as *Mycobacterium bovis* (6, 7). When mammalian tuberculosis (mTB) is endemic in the region, *M. bovis* is the most frequently identified mycobacteria in wildlife specimens. Nonetheless, in situations of low or nonexistent prevalence, the identification of NTM becomes more significant (4). Free-ranging wildlife can potentially encounter these environmental mycobacteria within their natural habitat, particularly during foraging and water consumption (4).

In Argentina, mTB is endemic in cattle in almost every region of the country (8, 9). Research efforts directed toward the surveillance of this disease in the local wildlife populations, particularly focusing on invasive alien species, are a recent development. Furthermore, besides the detection of *M. bovis* in both exotic and native species in Argentina, NTMs with relevance in public health and veterinary contexts were identified as well (10–13).

In Argentina, there are programs aimed for the reintroduction and protection of threatened native species in the region (14). Within these programs, there are instances where sample collections are feasible, in activities such as health check-ups prior to the release or translocation of the specimens, and during the capture of individuals for the placement of monitoring collars. Among the species encompassed within such conservation initiatives is the Giant Anteater (*Myrmecophaga tridactyla*). This species is actively engaged in both conservation and reintroduction efforts, as documented in studies by Jiménez-Pérez et al. (15) and Zamboni et al. (14). Furthermore, it holds a threatened status according to the Ministry of Environment and Sustainable Development (16). For this species, poaching is one of the main threats, and many babies are rescued and raised within these conservation programs (14, 15). Another threatened wildlife species involved in conservation programs is the Pampas Deer (*Ozotoceros bezoarticus*), which is considered endangered (16). The reasons for its decline include intense commercial exploitation (for skins and meat), poaching, habitat destruction and alteration, predation by dogs, competition with livestock, and disease transmission by introduced wildlife species (17). Similarly, the tapir (*Tapirus terrestris*) benefits from a Conservation Action Program (18) and holds a threatened status (16). Uncontrolled sport hunting and the reduction of forested areas are among the leading causes of its disappearance. Lastly, the collared peccary (*Pecari tajacu*) is involved in reintroduction programs (14, 19) and is also classified as a threatened species, with its primary threat being hunting (17).

Few reports are available where epidemiological surveillance of mycobacteriosis is conducted on samples taken from alive native wildlife, especially those with conservation risk, as in this study. Usually, sampling is carried out on tissue from deceased animals within surveillance programs, roadkill, or as part of population control efforts (4, 20). When sampling alive animals, especial conditions are needed, as the collection must be fast as the animals are anesthetized and also, sampling is not invasive most of the times.

Investigating the health condition of native wildlife in a certain region would help protect biodiversity in that ecoregion. This research aims to evidence the presence of NTM in free-ranging native wild animals with different degrees of conservation concern in Argentina.

2 Materials and methods

Between the years 2016 to 2021, the Laboratory of Tuberculosis Diagnosis of the Infectious Diseases Department in the Faculty of Veterinary Science at the University of Buenos Aires received 165 (each corresponding to only one animal: 104 Collared peccary, 31 Pampa's deer, 19 Tapir and 11 Giant anteaters) samples from anesthetized native mammals from 9 provinces of Argentina, including Buenos Aires, Chaco, Córdoba, Corrientes, La Rioja, Mendoza, Salta, Santiago del Estero y Tucumán as part of the health checks within conservation programs. The sampled animal species present in each province is shown in Figure 1. The anesthetic protocol adhered to the standard procedures of each institution during these procedures and was carried out by the institution's responsible veterinarian group, following guidelines that ensure animal's welfare (21). The received samples included bronchoalveolar lavages, endotracheal lavages, oropharyngeal swabs, nasopharyngeal swabs, and tracheal swabs. The conservation categories of each native wildlife species were determined in accordance with Resolution 316/2021 from the Ministry of Environment and Sustainable Development. The samples were kept frozen at−20°C until shipment and processing in the Laboratory of Tuberculosis Diagnosis (FCV-UBA).

2.1 Bacteriological culture

The bacteriological culture of the submitted samples was performed using the Löwenstein-Jensen medium. The Petroff decontamination technique was applied beforehand, treating rhe sample with NaOH (4%) and the sediment obtained is hen neutralized with HCl and placed in the sterile medium, as specified bu Jorge et al. (22). Cultures were incubated at 37°C for up to 12 weeks, and those with no bacterial growth were discarded as negative (22). Colonies compatible with mycobacteria growth were stained using the Ziehl-Neelsen technique for acid-fast bacilli (AFB) observation.

2.2 Molecular diagnosis

For the detection of the genre *Mycobacterium* in the isolates, DNA from the bacteriological cultures were obtained by thermal lysis. For this purpose, a colony was taken with a sterile 1 μL loop and suspended in 300 μL of sterile pyrogen-free distilled water in 1.5 mL RNase-free microtubes. Subsequently, the microtubes were subjected to 95°C for 40 min and centrifuged for 10 min at 12,000 rpm. DNA was kept frozen at −20°C until processing. This DNA was subjected to PCR amplification of the heat shock protein 65 kD

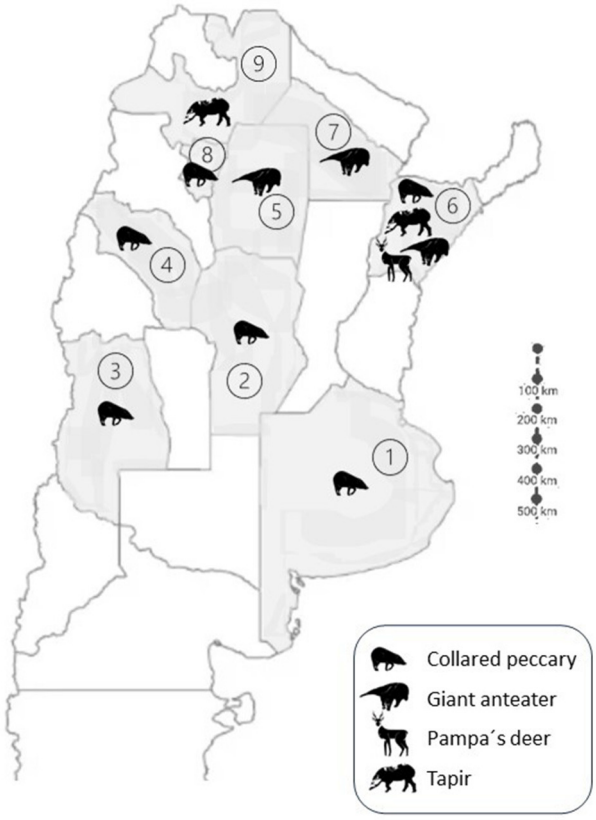

FIGURE 1
Partial Argentinean map showing the provinces where the native wildlife species are present. The different species are represented with the animal silhouette in each province. References: Provinces: 1. Buenos Aires; 2. Córdoba; 3. Mendoza; 4. La Rioja; 5. Santiago del Estero; 6. Corrientes; 7. Chaco; 8. Tucumán; 9. Salta.

(*hsp*65) using the primers TB11 (ACCAACGATGGTGTGTCCAT) and TB12 (CTTGTCGAACCGCATACCCT) and the cycling described by Telenti et al. (23). All PCR products were included in a 2% agarose gel stained with Ethidium bromide (0.5 μL/mL) and observed under a UV light. Those isolates shielding a band at 440 bp were considered positive. These isolates were further studied by sequencing and analyses. Some isolates from different species were selected for sequencing due to economic considerations, aiming to represent each species of wild animal and considering the quality of the band observed in the *hsp*65 PCR. The selected PCR products were purified using one of the following purification kits "Illustra DNA and Gel Band Purification Kit" (GE Healthcare, UK) or "GFX™ PCR DNA and Gel Band Purification Kit" (Cytiva, USA) following manufacturer's specifications. Purified products' quality was confirmed by quantification in a spectrophotometer at a wavelength of 260 nm (Nanodrop 2000, Thermo Scientific™, Thermo Fisher Scientific, USA).

For the detection of the *Mycobacterium avium* complex (MAC) members, PCR amplification of the insertion sequence *1245* was performed. Differentiation between species within the complex was achieved through amplification of insertion sequence *901*. For IS*1245*, amplification was performed using primers P1 (GCCGCCGAAACGATCTAC) and P2 (AGGTGGCGTCGAGGAAGAC) and the cycling described by Guerrero et al. (24). A band weighing 427 bp was considered a positive sample for MAC. All MAC-positive isolates were further studied for subspecies detection amplifying the insertion sequence *901*, using primers P1 (GGATTGCTAACCACGTGGTG) and P2 (GCGAGTTGCTTGATGAGCG) and the cycling described by Moravkova et al. (25). When a PCR product of 577 bp was observed, *Mycobacterium avium* subsp. *avium* was identified, while when no bands were observed *Mycobacterium avium* subsp. *hominissuis* was identified.

2.3 Sequencing and analyses

Sequencing was carried out in the Genomics Unit of the Institute of Biotechnology, in the Institute of Agrobiotechnology and Molecular Biology (IABIMO INTA-CONICET) for the Giant anteater, Tapir and some Pampas deer, and for the collared peccary by Macrogen sequencing service (Korea). The institution that provided sequencing changed during the years these animals were sampled, but both institutions performed the same sequencing, using a 16-capillary sequencer ABI3130xl (Applied Biosystems, Thermo Fisher Scientific, USA), using "Big Dye Terminator v3.1" (Cycle Sequencing Kit). The sequences that shielded good quality were compared to those in the National Center of Biotechnology Information using the Basic Local Alignment Search Tool (BLAST). Identification was based on similarities between our isolates and those in the database, identifying the best match considering the percentage of coverage and identity.

3 Results

A total of 27 NTM were detected from the 165 (16,36%) investigated samples. The frequency and identity of the *Mycobacterium* and the frequency in each animal species can be observed in Table 1. Twenty-seven *hsp*65 positive isolates were subjected to IS*1245* PCR and in 4 samples MAC was detected, all of them were IS*901* negative, being identified as *M. avium* subsp. *hominissuis*. From the remaining IS*1245* negative samples, 20 samples were sent for sequencing. Not all the *Mycobacterium* revealed a clear species identity and coverage when compared to those in the BLAST online database, being identified with the same percentage of identity and coverage as more than two species or only being identified as "*Mycobacterium* spp.". Those with no clear identification were kept as "*Mycobacterium* spp." for this work. In regards to the giant anteater, the species identified were: *M. avium* subsp. *hominissuis* (1/5), *M. terrae* (1/5), *M. gordonae* (1/5) and the rest were identified as *Mycobacterium.* spp (2/5). In regards to the tapir, *M. genavense* (1/6), *M. saskatchewanense* (1/6), *M. intracellulare* (1/6) and three other *Mycobacterium* spp were identified. Regarding the collared peccary, *M. avium* subsp. *hominissuis* (2/10), *M. terrae* (1/10), *M. kumamotonense* (1/10), *M. fortuitum* (1/10) and five other *Mycobacterium* spp. (5/10) were identified. Lastly, regarding pampas deer, *M. avium* subsp. *hominissuis* (1/4), *M. intracellulare* (1/4) and two other *Mycobacterium* spp. (2/4) were detected. The most frequent *Mycobacterium* species detected were *M. avium* subsp. *hominissuis*

TABLE 1 Frequency of the identified Mycobacteria in the native wildlife species.

Province	Animal species	Sample	Isolated	*n*
La Rioja	Collared peccary	Tracheal swab	*M. terrae*	2
			Mycobacterium spp.	3
Mendoza	Collared peccary	Tracheal swab	*M. avium* subsp. *hominissuis*	1
			Mycobacterium spp.	1
Tucumán	Collared peccary	Tracheal swab	*M. avium* subsp. *hominissuis*	1
			M. kumamotonense	1
			M. fortuitum	1
			Mycobacterium spp.	1
Corrientes	Giant anteater	Oropharyngeal swabs	*M. avium* subsp. *hominissuis*	1
			Mycobacterium spp.	1
	Tapir	Bronchoalveolar lavages	*M. genavense*	1
		Oropharyngeal swabs	*Mycobacterium* spp.	1
			M. saskatchewanense	1
	Pampa's deer	Lavages endotracheal	*M. avium* subsp. *hominissuis*	1
			M. intracellulare	1
			Mycobacterium spp.	2
Salta	Tapir	Bronchoalveolar lavages	*Mycobacterium* spp.	1
		Oropharyngeal swabs	*M. intracellulare*	1
			Mycobacterium spp.	1
Córdoba	Collared peccary	Tracheal swab	*Mycobacterium* spp.	1
Santiago del Estero	Giant anteater	Oropharyngeal swabs	*M. terrae*	1
			Mycobacterium spp.	1
Chaco	Giant anteater	Oropharyngeal swabs	*M. gordonae*	1
Total				27

and *M. terrae*. The animal species with the highest Mycobacteria detection was the giant anteater.

4 Discussion

Our study reportsa high NTM recovery rate (16,36%; 27/166) in samples from native wildlife species from different regions of Argentina. None of the sampled animals exhibited clinical signs associated with chronic disease prior to sample collection. Furthermore, from the time of sample collection until the writing of this manuscript, no health data were obtained from these animals. Therefore, it remains unknown whether they developed clinical signs or lesions at any point in their lives.

The animal species with the highest presence of NTM was the giant anteater (45,5%; 5/11). This high recovery of mycobacteria in the oral mucosa of this species might be associated with the way this animal feeds and the distinctive characteristics of its tongue, which is softer, wetter, and rougher, allowing it to adhere to objects before ingestion (26). The presence of NTM such as *Mycobacterium fortuitum* in this species has been reported previously (27). We did not find any literature describing MTBC infection in this species. This could be due to various reasons, such as limited research on the species, potential resistance of the species to pathogenic mycobacteria, or the possibility that environmental mycobacteria colonize the oropharyngeal mucosa, and potentially regulate or interfere with the colonization of pathogenic mycobacteria, directing the mucosal immune response as has been suggested by other authors in human medicine (28–30). More research is required to corroborate this statement for this species.

Tapirs showed a 32% (6/19) prevalence of NTM. This species is reported to be highly susceptible to both *M. bovis* and *M. tuberculosis* (31–34). Given the tapir's high susceptibility to *M. bovis* and the fact that they were moved from regions where mTB is endemic (7), a comparative intradermal tuberculin test (SICCT) was additionally performed, using both Purified Protein Derivatives (PPD) (bovine and avian) applied on the edge of the ear. This test was negative for both PPDs in all the cases. These results imply a higher sensitivity in detecting NTM from pharyngeal swabs and bronchoalveolar lavage samples through bacteriological culture compared to the SICCT. With these results, we support the assertion that the comparative SICCT, although it is recommended to detect *M. bovis,* is definitely an inadequate test for the detection

of NTM infections in tapirs, as observed by Marcordes et al. (32) in his study.

In the collared peccary, an incidence of 11,5% (12/104) of NTM was detected. In a study conducted in the same region, Brazil, on 330 samples of peccary lymph nodes, a 3% NTM was detected (35). There are reports describing susceptibility to *M. bovis* infection in this species, and they have been suggested as a possible reservoir for free-ranging animals in some areas of Brazil (35, 36).

The pampas deer is a threatened species in our territory and declines in its wild populations are reported annually (37). In this study, 13% (4/31) of NTM were identified. The *Cervidae* family is highly susceptible to mTB, both in free-ranging and captive animals (38). Several species of deer have been officially recognized as reservoirs of mTB in several countries across the globe (39–42). There are also reports that demonstrate the presence of NTM in this family (43).

Although the presence of NTM might interfere with the routine mTB diagnostic tests, this is more frequent in areas where TB prevalence in cattle is low. Other authors from Spain had reported *M. avium* subsp. *avium* and *hominissuis* and *M. nonchromogenicum* as the most common NTM identified in TST-reactors cattle (4). Another author reports that sensitization with *M. nonchromogenicum*, *M. intracellulare*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *hominissuis*, within others, could make animals react to the SITT (44). In Argentina, a study conducted by Oriani et al. (12), in which cattle were inoculated with NTM isolated from soils and wetlands, this NTM included *M. kansasii, M. nonchromogenicum, M. gordonae, M. arupense, M. phlei, M. fortuitum* and *M. peregrinum,* and showed that they may cause unspecific reactions, but that these reactions are not maintained over time (12).

As a limitation of this study, we mention the type of samples collected and analyzed for diagnosis. Given that the animals under study were alive, obtaining tissue samples was not feasible. The samples analyzed were restricted to those that could be collected during the examination of the oral and respiratory cavities, knowing that in the literature, the most representative samples for NTM and *M. bovis* detection are head, mediastinal, and mesenteric lymph nodes from deceased animals (37, 39).

The 16S ribosomal RNA and ***hsp***65 sequencing are both effective for identifying bacteria, particularly *Mycobacterium* species. ***Hsp***65 sequencing yields comparable results to the widely used 16S ribosomal RNA, as reported by various studies, including one conducted in our laboratory on NTM) in animal samples (10). Other authors also confirm that the use of either 16S ribosomal ARN or ***hsp***65 would allow the identification of *Mycobacterium* spp. And, in many cases, to the species level (45). Moreover, a combination of more than one sequence could strengthen the identification of the species, using a combination of at least three different sequences. Also, the use of multilocus sequence typing would improve the identification of mycobacterial species (46, 47). Additionally, poor quality or incorrectly identified sequences could limit the identification when compared against the BLAST database (4, 48).

The studied animals came from different provinces to Corrientes province, where they were relocated. These provinces are located far from each other and have different soil and climate conditions, but no significant clustering of NTM species in each region was observed. Among the Mycobacteria detected in this study, ubiquitous environmental bacteria were isolated, such as *M. avium*, *M. gordonae*, *M. terrae*, *M. fortuitum*, *M. kumamotonense*. These Mycobacteria can be isolated from soil, water and occasionally have been associated with disease in animals and humans (3, 49–55). *M. avium* has been isolated from several wild species (4) and MAH has already been reported in wild and domestic animals in Argentina (56). *M.intrcellulare* has been reported causing disease in a capybara subjected to stressful conditions and causing lesions similar to other pathogenic Mycobacteria (5). *M. gordonae* and *M. terrae* have been isolated from sputum samples in human patients with respiratory disease (3, 57), but there are no reports of these agents' causing disease in domestic and wild animals. *M. kumamotonense* has been documented in immunocompetent individuals with latent tuberculosis and patients with multiple spiculated pulmonary nodules without respiratory symptoms (58, 59). Other NTM such as *M. saskatchewanense* and *M. genavense* are found in clinical samples from humans in North America and Europe, acting as opportunistic pathogens in immunocompromised patients (60, 61). *M. genavense* has been reported in various wild and domestic animals, including birds, rabbits, cats, ferrets, snakes, and dogs (62–64). According to most authors, transmission to humans occurs through oral ingestion from contaminated water or close contact with infected animals. Additionally, a study in the Serengeti ecosystem, focusing on NTM, found *M. fortuitum* to be a prevalent species. This *Mycobacterium* was identified in cattle tissues and in the sputum of humans showing clinical signs suggestive of tuberculosis (20). NTM detected in this study have been previously reported in soil, water and cattle and wildlife in Argentina (12, 13, 65).

Emphasizing the importance of infections caused by NTM in human medicine is crucial. The prevalence of NTM in humans is increasing, and there is a belief that in certain industrialized countries, it might exceed the incidence of tuberculosis caused by MTBC (66). Additionally, it is known that NTM has developed resistance to most conventional antibiotics, making treatments ineffective and underscoring their profound impact (67). Although the interaction between human and wildlife is occasional in developed countries, in developing countries the human-wildlife interface is becoming increasingly frequent. Therefore, it is important to understand the distribution of mycobacteria in wildlife from different regions, since the information is very scarce. The active surveillance of wildlife reflects what is happening in the environment, which is the primary source of infection for both humans and coexisting animals (68).

The transmission of NTM between domestic and wild species can occur through direct contact but is largely mediated by shared environments (68, 69). The presence of NTM in free-ranging animals that share their environment with livestock highlights the need to differentiate mycobacteria species, because of the potential interference in diagnostic tests, to control mTB (4, 68, 70). In Argentina, there is a particular scenario where extensive livestock farming is the most frequent strategy, allowing domestic and wild animals to interact in the same environment, increasing the likelihood of disease transmission

between them, compared to more confined and intensive farming scenarios (56, 69). The adverse consequences associated with the introduction of livestock into habitats occupied by native fauna have been extensively documented, primarily due to the spread of infectious and parasitic diseases (71–78). The same scenario was observed in tapir (79), giant anteaters (80, 81), and peccaries (35). In regards to the peccaries, efficient transmission is also described between different wild species, such as the invasive exotic wild boar and the vulnerable native peccary (69–82).

Our study provides valuable insights into the presence and diversity of NTM in Argentina's native wildlife. This emphasizes the importance of active surveillance, highlighting potential risks to native species and advocating for conservation strategies to mitigate infectious diseases' impacts in shared environments.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MW043443; https://www.ncbi.nlm.nih.gov/genbank/, MW043444.

Ethics statement

Ethical approval was not required for the studies involving animals in accordance with the local legislation and institutional requirements because animals are wildlife in the context of Conservation and Translocation Programs. Fundación Rewilding Argentina has permits for all the activities they control. The Laboratory of Tuberculosis Diagnosis received the samples and no Ethics Committee is required. Written informed consent was not obtained from the owners for the participation of their animals in this study because this animals have no owners or where translocated from zoos or rescue facilities. Permits are not required as quarantine is mandatory and this tests are in the context of health check-ups.

Author contributions

SB: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing—original draft, Writing—review & editing. LP: Methodology, Writing—original draft, Writing—review & editing. IP: Methodology, Writing—original draft, Writing—review & editing. AR: Methodology, Visualization, Writing—review & editing. JP: Methodology, Visualization, Writing—review & editing. MM: Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Research was supported with UBACyT Projects (20020170200311BA; 20020130100082BA 20020190200309BA) and Fundación Rewilding Argentina own projects.

Acknowledgments

We thank veterinaries and volunteers of Fundación Rewilding Argentina. SB is career member of CONICET, Argentina.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Public Health

TYPE Brief Research Report
PUBLISHED 02 April 2024
DOI 10.3389/fpubh.2024.1358261

OPEN ACCESS

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RECEIVED 19 December 2023
ACCEPTED 04 March 2024
PUBLISHED 02 April 2024

CITATION
Castro-Rodriguez B, Franco-Sotomayor G, Rodriguez-Pazmiño ÁS, Cardenas-Franco GE, Orlando SA, Hermoso de Mendoza J, Parra-Vera H and García-Bereguiain MÁ (2024) Rapid and accurate identification and differentiation of *Mycobacterium tuberculosis* and non-tuberculous mycobacteria using PCR kits available in a high-burden setting. *Front. Public Health* 12:1358261. doi: 10.3389/fpubh.2024.1358261

Rapid and accurate identification and differentiation of *Mycobacterium tuberculosis* and non-tuberculous mycobacteria using PCR kits available in a high-burden setting

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Infections caused by mycobacteria, including *Mycobacterium tuberculosis* complex (MTBC) and non-tuberculous mycobacteria (NTM), are a major public health issue worldwide. An accurate diagnosis of mycobacterial species is a challenge for surveillance and treatment, particularly in high-burden settings usually associated with low- and middle-income countries. In this study, we analyzed the clinical performance of two commercial PCR kits designed for the identification and differentiation of MTBC and NTM, available in a high-burden setting such as Ecuador. A total of 109 mycobacteria isolates were included in the study, 59 of which were previously characterized as *M. tuberculosis* and the other 59 as NTM. Both kits displayed great clinical performance for the identification of *M. tuberculosis*, with 100% sensitivity. On the other hand, for NTM, one of the kits displayed a good clinical performance with a sensitivity of 94.9% (CI 95%: 89–100%), while the second kit had a reduced sensitivity of 77.1% (CI 95%: 65–89%). In conclusion, one of the kits is a fast and reliable tool for the identification and discrimination of MTBC and NTM from clinical isolates.

KEYWORDS

mycobacteria, *Mycobacterium tuberculosis*, atypical mycobacteria, PCR, clinical performance

Introduction

There are two main groups of mycobacteria of clinical importance and global distribution: (a) members of the *Mycobacterium tuberculosis* complex (MTBC), which cause tuberculosis; (b) atypical or non-tuberculous mycobacteria (NTM), which are ubiquitous and opportunistic microorganisms that may cause diseases in immunocompromised individuals and exhibit unusual abilities to survive in odd and extreme environmental conditions (1–3); therefore, their distribution appears to be environmentally defined and non-uniform, although it is still poorly

defined (4, 5). The group of NTM comprises any mycobacteria other than MTBC or *Mycobacterium leprae*, including over 190 species and subspecies with a wide range of abilities to cause pulmonary and extrapulmonary diseases (6, 7).

In high tuberculosis (TB)-burden countries, the detection and treatment of NTM rarely occur because of poor knowledge about NTM diseases and limited access to laboratory methods required for culture and molecular assays for species level identification. As these locations rely heavily on the microscopy for TB detection, NTM are commonly missed or mistaken for *Mycobacterium tuberculosis* when using acid-fast smears; consequently, patients are inappropriately treated with anti-TB drugs (3, 6, 8). Most countries do not report NTM diseases (3, 4, 6); therefore, the description of burden, trends, and associated risk factors depends on special studies, surveys, and sentinel surveillance programs (5). There has been an increasing trend in the incidence and prevalence of NTM diseases around the world in the last four decades; however, it varies across different regions (3, 5): in the United States, the prevalence of NTM cases increased from 2.4/100,000 in the 1980s to 15.2/100,000 in 2013 (9); Canada reported an increase from 4.9/100,000 in 1998 to 9.08/100,000 in 2010 (10); England, Wales, and Northern Ireland have shown an overall increase from 0.91/100,000 to 7.6/100,000 by 2012 (5); Brazil reported 0.25/100,000 prevalence in 2008 (3, 11); Taiwan reported an increase from 2.65/100,000 in 2000 to 10.17/100,000 in 2008 (12); South Korea shows an increase from 1.2/100,000 in 2003 to 33.3/100,000 in 2016 (5); and in China, the proportion of NTM cases increased from 15.6% in 2013 to 46.1% in 2018 (5); while in sub-Saharan Africa, the prevalence of pulmonary NTM diseases was 7.5% in a period between 1940 and 2016 (13). Additionally, a recent metadata analysis carried out from 2022 to 2024 has shown a continued increase in NTM isolation and disease across regions and in most countries in North America, Europe, and East Asia (14–17). According to one of those reports from 2022, the overall annual rate of change for NTM infection and disease per 100,000 persons/year was 4.0 and 4.1%, respectively (16, 17). Although no prevalence studies for NTM in Ecuador have ever been done, there are some recent case reports of pulmonary plastic surgery-associated infections caused by NTM (18, 19).

Tuberculosis is one of the major causes of death from a single infectious agent in the world and produces an estimate of 10.6 million ill people and 1.3 million deaths worldwide (20). It is a disease that mainly affects vulnerable population groups in countries with low socioeconomic development, worsening existing inequalities. While TB diagnosis and access to treatment improved in recent years in all regions of the world, the effect of the COVID-19 pandemic greatly disrupted the increasing trend of newly diagnosed TB patients and reports worldwide, from 7.1 million in the period 2017–2019 to 5.8 million in 2019–2020; however, an increase was observed to 7.5 million in 2022 (20, 21).

Either TB or mycobacteriosis diagnosis is still a challenge, especially in low- and middle-income countries (3, 20, 22). Bacilloscopy and mycobacterial culture are the gold standards for clinical diagnosis, although they are time-consuming tasks and require a high bacterial load and expertise to manipulate these microorganisms (1, 8, 23). Additionally, those methods do not allow accurate discrimination between MTBC and NTM (3, 6). As antibiotic treatments are totally different for those two groups of mycobacteria (2, 3, 20, 24), several methodologies for discrimination between MTBC or NTM are available, including biochemical tests (2, 3), Sanger sequencing (3, 6), or MALDI-TOF MS (8, 25–27). Furthermore, PCR protocols for the rapid discrimination of MTBC and NTM have been described. Moreover, there are several commercial PCR-based kits that became available recently, such as the Anyplex™ MTB/NTM Real-time Detection V2.0 (Seegene, South Korea) or Advansure™ TB/NTM Real-Time PCR Kit (LG Life Sciences, South Korea), with variable clinical performance depending on the brand and the study (28–32).

The aim of this study was to compare the clinical performance of two commercial PCR kits available in Ecuador for the rapid and accurate identification and differentiation of MTBC and NTM from the culture samples.

Materials and methods

Mycobacteria isolates included in the study

A total number of 109 mycobaceteria clinical isolates from the collection of "Centro Nacional de Referencia para Micobacterias" from "Instituto Nacional de Salud Pública e Investigación Leopoldo Izquieta Pérez" (INSPI) were included in the study.

A total of 50 of those cultures were previously characterized as *Mycobacterium tuberculosis* following the Pan-American Health Organization guidelines using the Kudoh – Ogawa method (33–35) and also characterized by 24 Mycobacterial Interspersed Repetitive-Unit Variable Number of Tandem Repeats (MIRU-VNTR) (36, 37).

The other 59 cultures corresponded to NTM isolated from skin purulent lesions using the Kudoh Ogawa method. Those cultures were characterized by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), as described elsewhere (27, 38), and included 29 *Mycobacterium abscessus* isolates, 9 *Mycobacterium farcinogenes* isolates and 19 *Mycobacterium fortuitum* isolates, 1 *Mycobacterium parafortuitum* and 1 *Mycobacterium novocastrense* isolates. Those NTM isolates are the most frequent within the area of study in Ecuador and are commonly associated with infections caused by plastic surgery (personal communication from clinical laboratories to the authors).

Mycobacteria culture heat inactivation and DNA isolation

Mycobacterial colonies were harvested from cultures, resuspended in Tris-EDTA (TE) buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and then inactivated at 95°C for 15 min. After heat inactivation, all samples were centrifuged for 5 min at 10,000 *g*, and the supernatant was directly used for molecular procedures, as reported elsewhere (39–42). The heat inactivation process was performed within the BSL2+ facility of the "Centro Nacional de Referencia para Micobacterias" from INSPI to prevent occupational exposure as it has been recommended (19).

qPCR using the VIASURE real-time PCR detection kit – MTBC + NTM (CerTest BIOTEC, Zaragoza, Spain) ("Viasure kit")

This IVD-CE marked qualitative real-time PCR assay is directed to the detection and differentiation of *Mycobacterium* genus, the

MTBC, and/or the specific differentiation of modern *M. tuberculosis* species (L2, L3, and L4 families) DNA from clinical strains and lower-track respiratory samples. The process is PCR-based with the use of specific fluorescent probes and primers for the amplification of conserved regions: a segment of the 16S rRNA gene for the detection of *Mycobacterium* representatives, insertion sequences IS*6110* and IS*1081* for the identification of mycobacteria that belong to the MTBC, and/or a fragment of the TbD1 deletion region, which allows the specific detection of *M. tuberculosis* strains classified as modern strains of the L2, L3, and L4 families. The format of the VIASURE RT-PCR Detection Kit used in this study contains 12 × 8-well strips, in which all reagents are lyophilized in each well for the RT-PCR assay: specific primers and probes, dNTPs, buffer, and polymerase, together with an Internal Control to discard inhibition of the polymerase activity. According to the manufacturer, after the addition of 15 µL of rehydration buffer to each well, a volume of 5 µL of the DNA sample was added to select wells, then 5 µL of reconstituted MTBC + NTM-positive control and 5 µL of MTB/NTM-negative control were added to separate wells for a final reaction volume of 20 µL in each well. A thermocycler (CFX96 from Bio-Rad) was programmed to detect signals (cycle threshold (Ct) ≤40) in the ROX channel (for 16S rRNA gene – *Mycobacterium* species), FAM channel (for IS*6110* and IS*1081* sequences – MTBC members), Cy5 channel (for TbD1 deletion region – "modern" *M. tuberculosis*), and HEX channel (for Internal Control), using the following PCR conditions: one cycle of 95°C for 2 min, followed by 45 cycles of 95°C for 10 s and 60°C for 50 s; fluorogenic data were collected after each cycle of this step (43).

qPCR using the *Mycobacterium* multiplex nucleic acid diagnostic kit (multiplex PCR – fluorescence probing) (Sansure Biotech, Changsha, People's Republic of China) ("Sansure kit")

This commercial PCR kit is designed to detect DNA from seven types of common clinical mycobacteria: *M. tuberculosis* (MTB), *Mycobacterium kansasii* (MK), *Mycobacterium avium* (MA), MF, *Mycobacterium abscessus* subsp. *abscessus* (MAA), *Mycobacterium abscessus* subsp. *massiliense* (MAM), and *Mycobacterium intracellulare* (MI). The detection is based on two kinds of PCR-technical principles: the first uses specific fluorescent probes to detect one target, and the second identifies another target by melting curve analysis, thus achieving simultaneous detection of both targets using the same fluorescence channel. The *Mycobacterium* Multiplex Nucleic Acid Diagnostic Kit (Multiplex PCR – Fluorescence Probing) has an open format; therefore, the volume of added reagents can be modified while maintaining the concentration of the reaction established by the manufacturer: each tube contained 13.28 µL of the MTB/NTM-PCR Mix, 0.23 µL of the MTB/NTM Enzyme Mix, and 1.5 µL of DNA from samples, while 1.5 µL of the MTB/NTM-positive control and 1.5 µL of the MTB/NTM-negative control were added to the reaction mixture in different tubes for a final reaction volume of 15 µL in each tube. The thermocycler was programmed as follows: one cycle of 50°C for 2 min; one cycle of 94°C for 3 min; and 45 cycles of 94°C for 10 s, 60°C for 20 s, and 75°C for 20 s (fluorogenic data were collected at the end of each cycle of this step); then, one cycle of continuous increase in temperature from 62°C to 75°C for fluorescence data collection for the melting curve analysis. The signal of specific fluorescent probes (Ct ≤ 39) is detected in the ROX channel for MF, in the FAM channel for MK, in the Cy5 channel for Internal Control, and in the HEX channel for MA, while DNA of MI is detected by the presence of a melting curve in the ROX channel (melting temperature peak (Tm peak): 70.5 ± 1°C), MAA is detected in the FAM channel (Tm peak: 69.5 ± 1°C), MAM is detected in the Cy5 channel (Tm peak: 68 ± 1°C), and MTB is detected in the HEX channel (Tm peak: 67 ± 1°C) (44).

Sanger sequencing for identification of NTM species

By means of Sanger sequencing and BLAST searches, NTM culture strains were evaluated for identification using the molecular markers 16S, *rpoB*, and *hsp65* genes. These three markers have been widely used to identify mycobacteria (45–49). The PCR amplification of the three genes was performed using GoTaq® Green Master Mix (Promega, Wisconsin, United States) following protocols described elsewhere (46, 47, 49). Amplification of fragments was confirmed by electrophoresis in 2% UltraPure™ Agarose (Invitrogen, California, United States) gels of 15 cm x 10 cm in 0.5X Tris-boric acid-EDTA buffer at 100 V for 3 h using a ladder 100 bp Plus Opti-DNA Marker (Cat. No.: G016, Applied Biological Materials Inc., British Columbia, Canada) for size determination. Amplicons were sequenced with the Sanger method and analyzed with the ABI 3500xL Genetic Analyzer from Applied Biosystems at the Service Department of Universidad de las Americas, Quito, Ecuador. The obtained sequences were curated using Geneious® v. 11.0.4 (Dotmatics, United Kingdom) and then proceeded to their identification by nucleotide-BLAST (NCBI, United States) search using default parameters.

Ethics statement

The access to this micobacteria collection was approved by IRB from Universidad de Las Américas (code 2024-EXC-001). All samples were anonymized, and no personal data of the patients were made available.

Results

Identification of MTBC cultures using the two commercial qPCR kits included in the study

The 50 MTBC cultures yielded a positive result for MTBC DNA detection either with the Viasure or the Sansure kit, showing a sensitivity of 100% (Tables 1, 2; Supplementary Data 1).

Identification of NTM using the VIASURE real-time PCR detection kit – MTBC + NTM

Fifty-six out of the fifty-nine NTM cultures previously characterized by MALDI-TOF MS were positive for *Mycobacterium*

TABLE 1 Clinical performance of the Viasure MTBC+NTM RT-PCR kit for the detection of the *Mycobacterium tuberculosis* complex.

		Reference method		Total
		Positive	Negative	
Viasure PCR kit	Positive	50	0	50
	Negative	0	0	0

TABLE 2 Clinical performance of Sansure *Mycobacterium* Multiplex RT-PCR kit for detecting *Mycobacterium tuberculosis* complex.

		Reference method		Total
		Positive	Negative	
Sansure PCR kit	Positive	50	0	50
	Negative	0	0	0

TABLE 3 Clinical performance of the Viasure MTBC+NTM RT-PCR kit for the detection of non-tuberculous Mycobacteria.

		Reference method		Total
		Positive	Negative	
Viasure PCR kit	Positive	56	0	56
	Negative	3	0	3

genus for the Viasure kit according to the manufacturer's instructions (Ct ≤ 40 in the ROX channel). The remaining three cultures were identified as MTBC cultures by the Viasure kit (Ct ≤ 40 simultaneously for ROX and FAM channels), which means a sensitivity of 94.9% (CI 95%: 89-100%) for detection of NTM (Table 3; Supplementary Data 1).

Those three NTM cultures identified as MTBC by the Viasure kit were subjected to Sanger sequencing to confirm the MALDI-TOF MS result. Two strains were identified as NTM through the *rpoB* and 16S markers, while one of the strains could not be further characterized by Sanger sequencing.

Additionally, the specificity of the Viasure kit was 94.9% (CI 95%: 89–100%) and 100% for MTBC and NTM culture identification, respectively.

Identification of NTM using the *Mycobacterium* multiplex nucleic acid diagnostic kit (multiplex PCR – fluorescence probing)

Thirty-seven out of the fifty-nine NTM cultures previously characterized by MALDI-TOF MS were positive for *Mycobacteria* for the Sansure kit, according to the manufacturer's instructions (see Methods). The remaining 19 NTM cultures were identified as negative samples, either for MTBC or NTM, which means a sensitivity of 62.7% (CI 95%: 50–75%) for the detection of NTM (Table 4; Supplementary Data 1).

As the Sansure kit is designed for the identification of certain *Mycobacteria* species, not including *M. farcinogenes*. *M.parafortuitum* and *M.novocastrense*, we considered the exclusion of the eleven

TABLE 4 Clinical performance of Sansure *Mycobacterium* Multiplex RT-PCR kit for detection of non-tuberculous mycobacteria.

		Reference method		Total
		Positive	Negative	
Sansure PCR kit	Positive	37	0	37
	Negative	22	0	22

TABLE 5 Clinical performance of Sansure *Mycobacterium* Multiplex RT-PCR kit for detection of non-tuberculous mycobacteria, including only these species: *M. kansasii, M. abscessus, M. abscessus* subsp. *massiliense, M. avium, M. fortuitum,* and *M. intracellulare*.

		Reference method		Total
		Positive	Negative	
Viasure NTM	Positive	37	0	37
	Negative	11	0	11

culture samples of that species for clinical performance analysis in Table 5. For the remaining forty-eight mycobacteria culture, thirty-seven were detected by Sansure kit, as mentioned above, which means that sensitivity of the Sansure kit for the detection of the NTM species indicated by the manufacturer was 77.1% (CI 95%: 65–89%).

Additionally, the specificity of the Sansure kit was 100% for either MTBC or NTM culture identification.

Reproducibility analysis

The reproducibility of both commercial PCR kits was assessed by running in triplicate all the non-matching samples compared to the standard method, as well as 20% of the matching results. We found 100% reproducibility for all samples analyzed for both the Sansure and Viasure PCR kits.

Discussion

Mycobacterial infections are a major public health issue worldwide due to different conditions related to these microorganisms (i.e., virulence, sensitivity, and resistance to antibiotics), the host (i.e., comorbidities and immunological status), and the environment where they develop (i.e., precarious living conditions, socio-economic inequity, and human migration) (40, 50, 51). Furthermore, the infrastructure, skills, and time needed to perform the microbiological procedures required for sampling, culturing, and identifying mycobacteria are negatively impacting its diagnosis (3, 4, 6). Additionally, proper identification of mycobacteria is fundamental for accurate treatment, as not only the antibiotic therapy varies between TB and mycobacteriosis but also depends on the NTM species (2, 3).

Therefore, fast and reliable identification and differentiation of infectious mycobacteria has become a critical priority in the field of microbiological diagnosis, and the implementation of PCR-based techniques has allowed the development of a variety of commercially available kits that perform identification assays

in a matter of hours (52–54). For instance, there are several reports addressing the clinical performance of some commercial kits, such as the Anyplex™ MTB/NTM Real-time Detection V2.0 (Seegene, South Korea), with reported sensitivities for MTB detection ranging from 71 to 86% and reported specificities ranging from 94.9 to 99%; while for NTM detection, the reported sensitivities ranged between 44.9 and 100% and the reported specificities ranged from 97.7 to 97% (29, 31, 32, 55). For the Advansure™ TB/NTM Real-Time PCR Kit (LG Life Sciences, South Korea), MTB detection sensitivity ranges between 78.1 and 96.2 and specificity ranges from 93.8 to 96.2%, while NTM detection sensitivity was in the range of 25–51.7% and specificity ranges between 97.8% and 98.3% (31, 32). All these cited reports worked with DNA extracted from clinical samples for the PCR-based assay and compared against culture results, which is considered the gold standard for mycobacterial diagnosis (29, 33). Although these reports endorse the use of PCR-based methods as suitable techniques for rapid mycobacterial detection, clinical performance evaluation studies are necessary as a wide range of sensitivity values were obtained, not only depending on the commercial brand but especially when comparing MTBC with NTM. To date, those reports support that, at least for NTM identification, PCR testing should be accompanied by microbiological analysis for a definitive diagnosis (29, 52, 54).

In this study, to the best of our knowledge, we present the first clinical performance evaluation of two commercial PCR kits recently available for MTBC and NTM identification. For rapid identification of MTBC cultures, both Viasure and Sansure kits are highly reliable tools with 100% sensitivity compared to gold standard methods. However, there was a slight reduction in MTBC identification specificity for the Viasure kit, although co-infection of MTBC and NTM not detected by MALDI-TOF MS cannot be totally ruled out.

By contrast, we found strong differences in the clinical performance of the Viasure and Sansure kits for the identification of NTM. The Viasure kit exhibited a great clinical performance with 94.9% (CI 95%: 89–100%) sensitivity and 100% specificity. However, even for the set of NTM species that the Sansure kit was designed for, the sensitivity value obtained was 77.1% (CI 95%: 65–89%). The importance of conducting these kinds of studies in different geographical settings, as the distribution of NTM species varies worldwide, has been emphasized. For instance, the NTM species included in this study are some of the most common in skin lessons associated with plastic surgery in Ecuador (personal communication to the authors). However, one of the kits evaluated in the study is not designed for the detection of three of those species, not within the most prevalent NTM species in other settings.

Considering that both commercial kits are also recommended for the direct detection of mycobacteria in clinical samples, we would anticipate poor clinical performance for the Sansure kit for NTM detection in clinical samples, while further experiments will be needed to address the clinical performance of the Viasure kit. This poor performance is actually and simultaneously the main limitation and further direction of our study. We are currently working in a study to address the clinical performance of these two PCR commercial kits for the detection of MTBC and NTM directly in clinical samples from TB and mycobacteriosis patients.

In conclusion, one of the PCR kits evaluated in this study (Viasure kit) is a fast and accurate tool for the identification and differentiation of MTBC and NTM from clinical isolates. This type of molecular test could help with the fast and affordable triage of clinical isolates in middle- to high-burden settings such as Ecuador, where differential diagnosis for TB and mycobacteriosis is a challenge.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

BC-R: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. GF-S: Data curation, Formal analysis, Funding acquisition, Investigation, Supervision, Methodology. AR: Data curation, Methodology, Writing – review & editing. GC-F: Writing – review & editing, Investigation, Methodology. SO: Supervision, Validation, Writing – review & editing, Investigation. JH: Supervision, Validation, Writing – review & editing, Resources. HP-V: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing. MG-B: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was funded by the Universidad de Las Americas (MED.MGB.23.01).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2024.1358261/full#supplementary-material

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frontiers | Frontiers in Public Health

TYPE Review
PUBLISHED 19 June 2024
DOI 10.3389/fpubh.2024.1410672

OPEN ACCESS

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RECEIVED 01 April 2024
ACCEPTED 10 June 2024
PUBLISHED 19 June 2024

CITATION
Zhang H, Tang M, Li D, Xu M, Ao Y and Lin L (2024) Applications and advances in molecular diagnostics: revolutionizing non-tuberculous mycobacteria species and subspecies identification.
Front. Public Health 12:1410672.
doi: 10.3389/fpubh.2024.1410672

Applications and advances in molecular diagnostics: revolutionizing non-tuberculous mycobacteria species and subspecies identification

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Non-tuberculous mycobacteria (NTM) infections pose a significant public health challenge worldwide, affecting individuals across a wide spectrum of immune statuses. Recent epidemiological studies indicate rising incidence rates in both immunocompromised and immunocompetent populations, underscoring the need for enhanced diagnostic and therapeutic approaches. NTM infections often present with symptoms similar to those of tuberculosis, yet with less specificity, increasing the risk of misdiagnosis and potentially adverse outcomes for patients. Consequently, rapid and accurate identification of the pathogen is crucial for precise diagnosis and treatment. Traditional detection methods, notably microbiological culture, are hampered by lengthy incubation periods and a limited capacity to differentiate closely related NTM subtypes, thereby delaying diagnosis and the initiation of targeted therapies. Emerging diagnostic technologies offer new possibilities for the swift detection and accurate identification of NTM infections, playing a critical role in early diagnosis and providing more accurate and comprehensive information. This review delineates the current molecular methodologies for NTM species and subspecies identification. We critically assess the limitations and challenges inherent in these technologies for diagnosing NTM and explore potential future directions for their advancement. It aims to provide valuable insights into advancing the application of molecular diagnostic techniques in NTM infection identification.

KEYWORDS

non-tuberculous mycobacterium, molecular diagnostics, whole genome sequencing, next-generation sequencing, drug resistance gene

1 Introduction

Non-tuberculous mycobacteria (NTM) encompass a diverse assembly of species distinct from the *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium leprae* (*M. leprae*). Identified initially in the late 19th century, NTM are characterized as Gram-positive, rod-shaped bacteria, often exhibiting branching or curved formations. The cell wall structure of NTM, rich

in lipids, phenolic glycolipids, and mycolic acids, is a key biological marker, rendering these bacteria acid-fast positive, similar to *Mycobacterium tuberculosis* (MTB), as demonstrated by staining techniques like the Ziehl-Neelsen staining method (1, 2). However, while useful, acid-fast staining alone cannot distinguish between MTB and NTM, necessitating further molecular diagnostic interventions for accurate identification. NTM is classified based on their growth rate in subculture as either rapidly growing mycobacteria (RGM), with a growth cycle of less than 7 days, or slowly growing mycobacteria (SGM), requiring 7 days or more to grow. To date, approximately 200 species of NTM have been identified, which can be further differentiated into various subspecies. The distinct clinical features and treatment responses of different subspecies play a crucial role in understanding the clinical management and therapeutic strategies for diseases associated with NTM (3, 4). The species diversity of NTM significantly varies across global regions, with the pathogenic potential of NTM species being geographically distinct. Additionally, in developed nations, the disease burden attributable to NTM infections now exceeds that of MTB (5–8).

NTM infections can manifest across multiple tissue and organ systems, such as the respiratory, central nervous, lymphatic, articular, and dermal systems. Notably, pulmonary infections account for roughly 90% of these cases (9, 10). The clinical manifestations of pulmonary infections caused by NTM are characterized by their nonspecific nature. Although chronic or recurrent coughing is almost universally reported among patients, there is variability in symptoms such as expectoration, fatigue, malaise, dyspnea, fever, hemoptysis, chest pain, and weight loss. In children aged 1 to 5 years, NTM primarily infects submandibular, cervical, or preauricular lymph nodes, indicating its capacity to also target tissues beyond the commonly affected systems (11, 12). Currently, approximately 80% of culture-confirmed NTM lymphadenitis cases are caused by the *Mycobacterium avium* complex (MAC) (13). Recent studies emphasize the evolving landscape of NTM species affecting lymph nodes, highlighting the need for advanced molecular diagnostics to accurately identify and tailor treatment strategies. Furthermore, studies reported a steady increase in the variety of NTM species isolated from lymph nodes, including *Mycobacterium lentiflavum* (*M. lentiflavum*) (14).

Accurate diagnosis and treatment of NTM infections necessitate a nuanced and individualized assessment of therapeutic risks versus benefits. The widespread environmental presence of NTM poses substantial challenges for clinicians in accurately identifying infections. Detecting NTM in non-sterile respiratory specimens does not necessarily indicate pathogenic involvement in pulmonary conditions, potentially indicating colonization, transient infection without clinical disease, or sample contamination. Furthermore, the pathogenic potential of NTM significantly depends on the host's immune status and the specific anatomical site of culture extraction. When NTM is isolated from sterile sites, including blood, tissues, cerebrospinal fluid, pleural fluid, or the brain, its presence almost always indicates clinical significance (15).

2 The critical role of molecular diagnostic techniques in enhancing the precision and efficiency of NTM species and subspecies identification

Traditionally, the identification of species within the NTM complex has relied on time-consuming biochemical tests and methods based on phenotypic characteristics, which often fail to provide accurate identification results. Pathogenic culture, despite its limitations, has historically been the gold standard for NTM identification and remains critical for drug sensitivity testing (DST) (16). While culture is indispensable for DST, molecular diagnostics play an increasingly crucial role in genotypic identification, offering more rapid and accurate results (4). The effectiveness of culture-based identification is markedly impacted by the growth kinetics of mycobacteria. Specifically, slow-growing strains of NTM often necessitate upwards of 7 days to yield discernible colonies. This contrasts sharply with molecular techniques, which can often identify NTM species within hours. Additionally, specific NTM species and subspecies, such as *Mycobacterium haemophilum* (*M. haemophilum*), *Mycobacterium marinum* (*M. marinum*), *Mycobacterium ulcerans* (*M. ulcerans*), and MAC, often cannot be identified through conventional culturing methods due to their stringent dependency on certain nutrients or specific culturing conditions (17–19).

To address these challenges, advancements in molecular diagnostics have revolutionized the identification of NTM infections, offering substantial improvements over traditional methods in terms of accuracy, diagnostic sensitivity, speed, and cost-effectiveness, especially in differentiating NTM species and genotyping. Several detection methods for NTM have been developed by adapting techniques originally designed for mycobacterial identification. While targeted single-gene sequencing effectively identifies most NTM species, the distinction at the subspecies level often requires multigene sequencing approaches (20, 21). Table 1 summarizes the comparison of DNA-based molecular techniques for identification of genetically closa NTM species, underscoring the critical role of these diagnostics in the precise detection of NTM (22–54).

3 Repetitive sequence-based methods in NTM identification

Repetitive sequence-based methods are widely utilized in molecular biology for the accurate identification of NTM. Leveraging the substantial variability inherent in the repetitive regions of bacterial genomes, these techniques distinguish between strains through comparative analysis of sequence differences.

3.1 Repetitive sequences involving NTM

Insertion sequence (IS) is a compact, mobile genetic element that predominantly encodes for transposition and regulatory functions. Its integration into specific genomic loci can disrupt gene functionality and alter the expression of nearby genes. Investigations across various NTM species have revealed multiple distinct IS types, underscoring their pivotal role in molecular diagnostics and contributing to genomic diversity. Among these IS elements, IS*1245* and IS*1311* are closely associated with the molecular typing of MAC strains (55). In addition to IS*1245* and IS*1311*, the MAC genome includes other insertion sequences. These insertion elements include IS*900*, present in *M. avium* subsp. *paratuberculosis* (MAP), IS*901* in *M. avium* subsp. a*vium* (MAA), IS*902* in *M. avium* subsp. s*ilvaticum* (MAS), and those yet to be thoroughly investigated in *M. avium* isolates (21, 56, 57). IS*900*, IS*901*, and IS*902*, of the reported IS elements thus far, have

TABLE 1 Comparison of DNA-based molecular techniques for identification of genetically closa NTM species.

Methods			Throughput	Resolution	Sensitivity	Specificity	Depth	Advantage(s)	Limitations	References
Repetitive sequence-based methods	MLVA	MIRU-VNTR	Poor	High	84.8% in *Mycobacterium ulcerans*	High	subspecies level	Fast, easy to perform, sensitive, highly reproducible, and discriminative; more discriminatory than IS6110- RFLP for NTM isolates with low copy no. Of IS6110; well suited for largescale, genetic, or evolutionary investigations; digitized results; can be performed directly on cell lysates; applicable for typing of NTM	The impact of VNTR marker choice, absence of uniform standards, the essentiality of advanced bioinformatics expertise and analytical instruments, and the need for high-integrity DNA.	(22–27)
	Rep-PCR		Poor	Moderate	Moderate	High	subspecies level	Commercially available;highthroughput automated system for typing of many NTM species; achieves higher level of discrimination than MIRU-VNTR typing for *M. avium* and provides better reproducibility	Necessity for process refinement and establishment of standard protocols, requirement for comprehensive bioinformatics expertise and advanced analytical technology, associated with significant expenditure.	(27–32)
Non-repetitive sequence-based techniques	Gene sequence analysis	Hybridization Probes	Poor	High	High	High	subspecies level	This method does not require a PCR amplification step and enables rapid detection and diagnosis	Relies on specific primers, limiting its applicability across diverse bacterial species.The clinically important *Mycobacterium abscessus* could not be identified	(33–35)
		LPA	Poor	High	High	High	subspecies level	This method has a low cost and no need to purchase special equipment	Limitations may arise due to incomplete coverage of NTM species, potential cross-reactivity, and the requirements for specialized equipment and technical expertise.	(36, 37)
		DNA microarrays	High	High	98.8%	100%	subspecies level	The detection method has fast detection speed, accurate results, high throughput and high degree of automation	Relies on specific primers, limiting its applicability across diverse bacterial species.	(38)
		REBA	Poor	High	87.61%	83.33%	subspecies level	The assay has high sensitivity, specificity and stability	Relies on specific primers, limiting its applicability across diverse bacterial species.	(39)
		PCR-RFLP	Poor	Moderate	High	Moderate	subspecies level	Does not require specialized equipment	Subject to the limitations of primers and restriction enzymes, this method involves tedious procedures, is restricted by enzyme recognition sites, and necessitates high-quality DNA.	(27, 40–42)
	Genome analysis	PFGE	High	High	High	Moderate	subspecies level	Inexpensive; data analysis easier than with REA	Characterized by significant equipment and technical demands, this method is expensive, time-intensive, not suited for high-throughput applications, and needs high-quality DNA.	(27, 43–45)
		RAPD	Moderate	High	Moderate	Moderate	subspecies level	Can be performed on unknown DNA sequence	Suffers from low resolution, inconsistent repeatability, reduced specificity, reliance on primers, and difficulty in interpreting outcomes.	(27, 46)
		AFLP	High	High	Moderate	High	subspecies level	Broad range of possible adjustments to improve discriminatory power of the method	Involves intricate procedures, is expensive, dependent on primers, requires complicated data analysis, and is difficult to standardize.	(27, 47, 48)
		LSP	High	High	Moderate	Moderate	subspecies level	Deligotyping is a very sensitive and efficacious approach for rapid screening of clinical isolates of NTM. The method is also well suited for constructing robust phylogenetic relationships	Dependent on the availability of a reference genome, limited applicability to various strains, and requires high-quality DNA.	(22, 49)
		WGS	High	High	High	High	subspecies level	May be performed directly on clinical samples (metagenomic approach); provides information on (nearly) the entire genome; allows detection of different genetic variants within the same population	Demands advanced equipment and technical proficiency, involves intricate data processing and analysis, is expensive, time-intensive, and necessitates high-quality DNA.	(27, 36, 50, 51)
	NGS	mNGS	High	High	High	High	subspecies level	Can be performed on unknown DNA sequence, Detects previously unknown mutations	Characterized by significant equipment and technical demands, this method involves complex data processing and analysis, is expensive, and exhibits low sensitivity for detecting target pathogens.	(52–54)
		tNGS	High	High	High	High	subspecies level	This assay has high throughput, strong targeting, low detection cost, and targeted detection of known pathogen subtypes and different drug resistance genes	Involves complicated data processing and analysis, is expensive, time-intensive, incapable of detecting novel pathogens, and necessitates a well-defined reference genome for probe design.	(53, 54)

MLVA, multilocus variable number of tandem repeat analysis; MIRU-VNTR, mycobacterial interspersed repetitive unit-variable number tandem repeat; Rep-PCR, repetitive element palindromic PCR; LPA, line probe assay; REBA, reverse blot hybridization assay; PCR-RFLP, PCR-restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; LSP, large sequence polymorphism; WGS, whole genome sequencing; mNGS, metagenomic next-generation sequencing; tNGS, targeted next-generation sequencing.

10.3389/fpubh.2024.1410672

been widely used to identify and differentiate various MAC strains (58–60). Extending **b**eyond MAC, there are numerous IS used for NTM epidemiological studies, highlighting the expansive utility of IS in the broader context of NTM research. These IS include IS*1395* in *M. xenopi*, IS*1511*/IS*1512* in *Mycobacterium gordonae* (*M. gordonae*), IS*1407* in *M. celatum*, IS*6120* and IS*2404* in *Mycobacterium smegmatis* (*M. smegmatis*), IS*2606* in *M. ulcerans*, *M. lentiflavum*, and *Mycobacterium kansasii* (*M. kansasii*), as well as IS*1652* in *M. kansasii* (61–66).

Trinucleotide repeat sequence (TRS) is ubiquitously present across bacterial genomes, varying in quantity, and serve as a pivotal tool for bacterial genotyping. Within the genomes of mycobacteria, the majority of these TRS are located within the genes of the Pro-Glu (PE) and PPE families (67). The PGRS-restriction fragment length polymorphism (RFLP) typing technique, which relies on the PGRS sequences, involves cloning these sequences into the recombinant plasmid pTBN12 to serve as probes for species identification. This approach has identified PGRS sequences across various NTM species, thereby facilitating the differentiation of strains such as *M. kansasii* and *M. ulcerans* through similar PGRS-RFLP typing methods. This advancement underscores the utility of TRS in the molecular diagnostics landscape, significantly enhancing the resolution of mycobacterial genotyping and contributing to our understanding of NTM infection identification (21).

Enterobacterial repetitive intergenic consensus (ERIC), characterized as imperfect palindromic sequences of approximately 126 bp, are predominantly distributed within the genomes of Gram-negative bacteria. The variability of ERIC sequences has facilitated the development of a novel genotyping approach, known as enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) typing (68). ERIC, a derivative of the RAPD technique, proves to be informative when utilized for targets beyond *Enterobacteria* (69). This method has been extensively applied to assess the genetic diversity among various species of Mycobacteria, including MTB, *M. gordonae*, *Mycobacterium intracellulare* (*M. intracellulare*), *Mycobacterium szulgai* (*M. szulgai*), *Mycobacterium fortuitum* (*M. fortuitum*), *Mycobacterium chelonae* (*M. chelonae*), and *Mycobacterium abscessus* (*M. abscessus*) (70–73). ERIC-PCR typing has significantly enhanced insights into the molecular epidemiology and phylogenetic relationships of these species, improving our comprehension of their transmission dynamics and pathogenic potential (74).

3.2 Repetitive sequence-based methods

Multilocus variable number of tandem repeat analysis (MLVA) characterizes tandem repeat regions dispersed across the NTM genome. These regions mirror the polymorphic minisatellites found in eukaryotic genomes. The variable number of tandem repeat (VNTR) loci encompasses five major polymorphic tandem repeat (MPTR) sequences (MPTR-A to E) and six exact tandem repeat (ETR) sequences (ETR-A to F) (75). MPTR sequences, comprising unique 10 bp sequences separated by 5 bp intervals, are identified across various NTM species, including *M. gordonae*, *M. kansasii*, and *M. szulgai* (76). MPTR sequences have been demonstrated to facilitate RFLP typing of *M. kansasii*, utilizing MPTR sequences as probes (66, 77). Supply et al. (78) introduced an optimized 24-locus mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) typing scheme, incorporating 12 loci previously identified. This expanded 24-locus format offers enhanced phylogenetic insight, establishing it as a standard method for typing the MTBC. MIRU-VNTR analysis is extensively applied in NTM typing, notably within *M. avium*, where the identification of multiple loci has revealed substantial discriminatory capability. In the case of *M. intracellulare*, up to 45 potential loci have been identified, with 7 exhibiting high variability, making them suitable for differentiation purposes. Additionally, 13 loci have been employed in the study of isolated *M. ulcerans*, further evidencing the utility of MIRU-VNTR analysis in nuanced differentiation and identification (24, 79).

Repetitive element palindromic PCR (Rep-PCR) represents a commercialized, high-throughput, automated system designed to genotype various species of mycobacteria by leveraging the variability in repetitive sequences scattered across bacterial genomes. This method amplifies repetitive fragments within non-coding sequences and separates them using a microfluidic electrophoresis chip (28). The NTM genome is characterized by various repetitive elements, including IS*6110*. The variability in sequence copy numbers and configurations yields unique genomic fingerprints. Rep-PCR leverages this to generate distinct profiles, differentiating closely related species or strains via subtle pattern shifts. Although some NTM species conserve specific repeating elements, others exhibit significant variations. This combination of conservation and variability aids in identifying NTM at both the species and strain levels with precision. Rep-PCR has been applied to the genotyping of *M. abscessus*, and its typing patterns have been compared with those obtained via pulsed-field gel electrophoresis (PFGE). The results demonstrated a 90% concordance in the typing of identical strains between Rep-PCR and PFGE analyses, suggesting that Rep-PCR may offer superior discriminatory power over PFGE (80). In the typing of various NTM species, Rep-PCR demonstrates enhanced repeatability relative to random amplified polymorphic DNA (RAPD) analysis. Furthermore, Rep-PCR offers superior discriminatory power compared to MIRU-VNTR typing for the MAC, underscoring its potential to improve resolution in NTM infection molecular diagnostics (72, 81).

4 Non-repetitive sequence-based methods in NTM identification

Non-repetitive sequence-based methods, while serving as a cornerstone in genetics, evolutionary biology, and biological research, have also been increasingly adopted in the molecular diagnostics of infectious diseases, including the identification of NTM infections. These methods facilitate the precise differentiation of NTM species, which are crucial for tailored therapeutic strategies. Contrary to repetitive sequences, non-repetitive sequences encompass gene-coding regions, regulatory elements, and functional sequences that, although not as prevalent as repetitive elements in the genome, play pivotal roles in genetic diversity and function. The analysis of non-repetitive sequences, through sequence alignment and specific gene or fragment sequencing, is pivotal for detecting genetic variations within NTM species.

4.1 Gene sequence analysis

Gene sequence analysis stands as a pivotal bioinformatics approach for examining and decoding the DNA sequences within

genomes, playing an essential role in the identification and study of NTM infections. By leveraging computational algorithms and bioinformatics tools for DNA sequence alignment, assembly, annotation, and analysis, this approach elucidates the NTM genome's structure, function, and evolution. It is instrumental in accurately distinguishing NTM species and subspecies, essential for precise treatment and management. Furthermore, genomic insights not only enhance our understanding of NTM biodiversity but also illuminate its public health implications, including the emerging patterns of antibiotic resistance and the geographical distribution of infections. This knowledge is imperative for informing public health strategies and interventions aimed at preventing and managing NTM outbreaks. Single nucleotide polymorphism (SNP) typing has shown low levels of homogeneity, making it a valuable tool for differentiating between species and thus has been employed in the identification of mycobacterial species. Technological advancements now enable the use of molecular beacons to identify single nucleotide substitutions, facilitating simultaneous analysis of multiple SNP loci. Gene sequence analysis leveraging SNP typing facilitates the identification of a broad spectrum of mycobacterial species, encompassing members within the MTBC, and enables the detection of various resistance markers (21, 82). Gene sequence analysis methods yield better results with the analysis of multiple loci. Multilocus sequence typing (MLST), which involves sequencing allele groups to differentiate species, is extended significantly in mycobacterial identification. Tools like PubMLST and mlstverse cater to the nuanced demand for subspecies-level identification (83, 84). PubMLST, combining conventional and ribosomal MLST, facilitates comprehensive bacterial identification. By accumulating and integrating genomic sequences from MLST, enhanced typing can be linked to prognosis and treatment resistance in emerging subspecies. This approach has effectively distinguished members of the MAC and fast-growing mycobacteria, although some studies indicate that MLST alone may not suffice for precise strain differentiation (43, 55, 85).

Initially, the utilization of DNA probe-based techniques to identify partial gene fragments represented a common technique in molecular biology. Depending on the type of target nucleic acid, a variety of amplification methods are employed based on their specific advantages, including conventional PCR for its simplicity and cost-effectiveness, real-time PCR for its quantitative capabilities, nucleic acid sequence-based amplification for high sensitivity, loop-mediated isothermal amplification for rapid and equipment-free amplification, and transcription-mediated amplification for its high amplification efficiency (86–90). Gene probe assays are available in two primary variants. Direct nucleic acid testing (NAT) traditionally required a substantial amount of bacterial material, although recent advancements in sensitivity allow for effective analysis with reduced bacterial loads. Incorporating an amplification step in nucleic acid amplification tests (NAATs) allows for the direct detection of bacterial DNA in clinical samples. The market offers a diverse array of commercial nucleic acid probes, which, when combined with the aforementioned amplification methods, enhance the detection and differentiation capabilities for a wide range of mycobacteria (encompassing both MTBC and NTM), with target sequences often including rRNA, 16S rRNA, and the 16S-23S rRNA spacer region (65, 91, 92). The line probe assay (LPA) employs a technique where target DNA is amplified using biotin-labeled specific primers. Subsequently, the amplified product is denatured and hybridized with specific oligonucleotides affixed to a nylon membrane. The results are then visualized through enzyme-linked immunochromatography. Notable LPA kits include the INNO-LiPA Mycobacteria v2 (Fujirebio Europe, Belgium), GenoType Mycobacteria CM, GenoType Mycobacteria AS, and GenoType NTM-DR kits (Hain Life Sciences, Germany), along with the Speed-oligo Mycobacteria (Vircell, Spain). The GenoType NTM-DR kits not only facilitates the identification of commonly encountered NTM to the species level, such as MAC, *Mycobacterium abscessus* complex (MABC), and *M. chelonae*, but also enables the detection of resistance to macrolides. This is achieved through the identification of mutations in the *erm41* gene and *rrl* gene, as well as resistance to aminoglycosides by detecting mutations in the *rrs* gene (37). However, in clinical mycobacterial identification laboratories, many still rely on commercial single-stranded DNA nucleic acid probe technology. This approach typically offers high species identification accuracy, with results available in a short timeframe (less than 24 h), facilitating rapid identification (93). The reverse blot hybridization assay (REBA) operates on the principle of affixing numbered oligonucleotide probes to a nitrocellulose or nylon membrane. These probes are then hybridized with biotin-labeled PCR amplification products. The presence of a colored signal at specific positions on the membrane strip indicates successful hybridization of the probe to the DNA fragment. Wang HY et al. conducted a comparative analysis of PCR-REBA with real-time PCR and RFLP techniques (94). Their findings demonstrated that PCR-REBA delivers highly sensitive and specific results in identifying NTM and distinguishing between NTM species from mycobacterial liquid cultures. DNA microarrays present a compelling alternative for conducting high-throughput analyses of multiple genetic markers concurrently, distinguishing themselves from traditional methods reliant on predefined probes. These chips can be tailored to target specific regions of interest within bacterial genomes, including conserved sequences typically associated with NTM species. This customization enables a more exhaustive analysis, facilitating the detection of genetic variations and the differentiation of closely related species. Despite the potential for elevated costs, the versatility and efficiency of DNA microarrays are increasingly appealing to clinical laboratories specializing in mycobacterium identification. Research has explored the utility of DNA microarrays in NTM identification (95, 96). With ongoing technological advancements driving down costs and enhancing performance, DNA microarrays are poised to become indispensable tools for NTM monitoring and research, addressing critical challenges in clinical settings and aiding in the development of more effective management strategies.

Currently, PCR-RFLP has been effectively employed in the identification of NTM, marking a significant advancement in gene sequence analysis. This method integrates PCR amplification, restriction enzyme digestion, and electrophoresis to generate species or strain-specific profiles. For the identification of NTM species using this approach, the process begins with the PCR amplification of the *rpoB* gene and the *hsp65* gene, resulting in PCR products. These are then subjected to digestion with specific restriction enzymes, such as MspI, HaeIII, and BstEII, followed by analysis using agarose gel electrophoresis. By employing additional restriction enzymes, this technique can further differentiate members of the MABC into subspecies levels (97).

4.2 Genome analysis

Genome analysis transcends basic gene sequencing, offering a detailed exploration of an organism's full genome, including gene organization, structure, and overall genomic context. This inclusive strategy provides intricate, high-resolution insights into both coding and non-coding regions and regulatory elements, shedding light on genetic variations, evolutionary links, and functional attributes. Consequently, it delivers a nuanced understanding of strain characteristics and their interconnections.

PFGE uses restriction enzyme digestion to fragment chromosomal DNA from different mycobacterial strains, producing unique fingerprint patterns that are visible under ultraviolet light after gel electrophoresis. The technique applies periodically alternating electric fields to direct DNA fragments, facilitating the separation of large molecules more effectively. A principal advantage is its capacity to separate larger DNA fragments, beyond the 50 kb limit of conventional unidirectional electrophoresis, by employing rare-cutting restriction enzymes. PFGE is characterized by its high repeatability and discriminatory capacity. Despite subtle strain variabilities, it proves highly effective in typing diverse NTM species, demonstrating particular success with slow-growing strains such as *M. kansasii*, MAC, and *M. avium* (98). However, it's important to acknowledge that PFGE involves significant costs, demands extensive technical expertise, and has a lengthy turnaround time, sometimes extending to 5 days. Like RFLP, PFGE also necessitates access to a comprehensive database of high-quality DNA sequences for reference (99).

Randomly amplified polymorphic DNA (RAPD), or arbitrary primed PCR (AP-PCR), is a technique independent of prior DNA sequence knowledge. Utilizing a single, arbitrarily chosen primer of 5 to 50 bp, it generates strain-specific DNA profiles by binding at sites with full or partial template DNA matches (100). Despite its high discriminative power, RAPD is limited by its poor reproducibility. Furthermore, it is generally believed that the observed differences between strains are more attributable to technical and procedural variations inherent to the method rather than true genetic polymorphism (101). Despite its limitations, RAPD-especially with multiple primer combinations-has been effectively used for NTM strain analysis. The polymorphism of DNA profiles from these combinations matches or exceeds those from PFGE. Moreover, RAPD's application extends to genotyping *M. abscessus* and *M. chelonae*, which are prone to spontaneous fragmentation during gel electrophoresis, thus complicating PFGE assessment (102, 103). Similarly, this method has been used for typing strains of *Mycobacterium phocaicum* (*M. phocaicum*), *M. gordonae*, *M. szulgai*, and *Mycobacterium malmoense* (*M. malmoense*) (104–107).

Amplified fragment length polymorphism (AFLP) analysis represents a PCR-based genotyping technique that utilizes dual restriction enzymes for DNA digestion: one rare cutter and one frequent cutter, recognizing sites of 6 bp and 4 bp, respectively. Following digestion, the resultant DNA fragments are ligated to double-stranded adaptors ranging from 10 to 30 bp, which are complementary to PCR primers. This setup facilitates the selective amplification of fragment subsets. AFLP analysis has emerged as a novel approach for typing NTM species, including members of the MAC and *M. hemophilum* (27). Additionally, AFLP has proven effective in distinguishing between closely related species, such as *M. marinum* and *M. ulcerans*, offering a refined tool for microbial identification and strain differentiation within the context of molecular diagnostics (21).

Large sequence polymorphism (LSP) analysis, a key molecular marker for examining genetic diversity in mycobacteria, depends on pre-existing sequence knowledge and often demands significant DNA quantities. It utilizes targeted PCR genotyping for focused studies and microarray technology for extensive genomic screenings. While microarray technology provides thorough genomic insights, it has discernible limitations. For instance, some platforms cannot detect deletions smaller than 350 bp, potentially compromising the sensitivity of NTM infection identification (108). This limitation underscores the need for integrating or developing more refined molecular diagnostic tools capable of detecting smaller genetic variations. Furthermore, the challenge of cross-hybridization among similar sequences necessitates the optimization of probe design and hybridization conditions, particularly for the non-repetitive segments of bacterial genomes, to enhance the method's applicability and accuracy in distinguishing between NTM subtypes. LSP analysis has been employed to identify differences between various subtypes within the MAC and *M. abscessus* (109).

Whole genome sequencing (WGS) represents a novel and highly precise method for identifying and characterizing various species of mycobacteria. Offering superior resolution compared to techniques such as targeted PCR genotyping and microarray analysis, WGS enables the differentiation of NTM subspecies down to their specific evolutionary branches, while also providing comprehensive genomic information. WGS provides unparalleled accuracy and precision in identifying genetic variances between strains by detecting almost all markers utilized in the genotyping methods mentioned previously. Its utility spans from offering comprehensive data at the global (population-wide), local (community), and individual (single patient) levels to yielding profound insights into the pathogens. It facilitates the precise identification of new NTM species and aids in predicting the virulence genes of NTM, laying a foundation for targeted therapy (50, 51). For example, WGS has successfully distinguished between closely related strains of *M. ulcerans* and *M. marinum*, providing insights into their evolutionary paths and virulence factors. Furthermore, its application in the study of MABC outbreaks has revealed specific transmission chains, highlighting its potential in public health surveillance and response (110, 111). Large-scale studies have validated the efficacy of WGS in mapping outbreaks and elucidating transmission pathways (112, 113). Compared with WGS, traditional methods such as PFGE and RFLP analysis often result in imprecise clustering, potentially missing fine-scale genetic variations critical for accurate pathogen identification and outbreak tracking. Furthermore, WGS has the capability to forecast undetected NTM species, thus facilitating the provision of timely and suitable interventions for emerging infections (114–117).

4.3 NGS

In recent years, NGS has significantly advanced our ability to harness comprehensive genetic information, especially via WGS. These technologies excel in pathogen detection with notable specificity, even amidst complex or sparse datasets, as demonstrated by metagenomic DNA analysis. Metagenomic NGS (mNGS) emerges as a superior, high-throughput method for DNA/RNA sequencing, outperforming

traditional Sanger sequencing by offering quicker processing, reduced costs, and enhanced sensitivity. NGS markedly accelerates the breadth of genomic or transcriptomic analyses, substantially boosting sequencing speed and efficiency (118). This enhanced depth and coverage are particularly valuable in NTM infection identification, as they facilitate the detection of low-frequency mutations or variants that might be missed by other methods (119). In bacterial evolution analysis, NGS shows superior advantages, including applications in phylogenetic analysis, speculation on species origins, and predicting microbial communities (120–122). NGS encompasses diverse approaches, including mNGS and targeted NGS (tNGS), each offering distinct advantages and applications across various contexts.

mNGS represents a broadly unbiased sequencing technique, capable of sequencing the genomes of a wide array of microbes present in a sample, although its efficacy can be influenced by sample quality and sequencing depth. This approach offers significant advantages for organisms like NTM, which are notoriously difficult to culture and identify. Crucially, mNGS eliminates the need for prior knowledge of the microbial composition within a sample, allowing for direct DNA extraction and sequencing from the specimen. While circumventing the limitations and biases inherent to traditional culturing methods, it's important to acknowledge scenarios where traditional methods may still offer valuable insights, particularly in understanding microbial growth characteristics and antibiotic susceptibility. mNGS facilitates the detection of microbial nucleic acids across a variety of specimen types and enables rapid identification of multiple pathogens, with numerous cases of NTM infections being diagnosed through this method (50, 52, 53).

Moreover, mNGS is adept at detecting low-abundance microbes, including NTM, making it essential for a comprehensive understanding of microbial communities within samples. mNGS exhibits the capability to uncover novel or hitherto unrecognized microbial species. In a study by Dougherty et al. (123), the utility of metagenomic analysis in identifying mixed infections was further corroborated. Their study underscored the prowess of shotgun metagenomics, not only for sequencing DNA from uncultured samples, but also for elevating mixed infection detection via target-specific amplification. This highlights its capability to refine microbial identification techniques. Thus, metagenomic analysis could signify a breakthrough in diagnostics, especially for multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains.

tNGS is a high-throughput sequencing method focused on specific genes or genomic regions, playing a pivotal role in the identification and study of NTM. This technique begins with the alignment of sequenced DNA against NTM sequences from curated reference databases, a critical step that enables the precise identification of specific microbial species and subspecies within the sample. Subsequently, by analyzing sequence variations, tNGS can elucidate critical information such as the NTM subtype, virulence factors, and resistance genes (124, 125). By focusing sequencing efforts on targeted genes or genomic regions of interest, tNGS not only reduces costs and turnaround times compared to WGS but also enables the rapid identification of resistance genes and virulence factors, crucial for tailoring treatment strategies in NTM infections. This streamlined focus makes tNGS an invaluable tool in the nuanced field of NTM research, offering precise insights into microbial genetics with efficiency (126, 127). While tNGS offers significant advantages in terms of cost and speed, it's important to acknowledge that by focusing only on predetermined genomic regions, it may miss novel or unexpected variants outside these regions, potentially limiting the comprehensive understanding of microbial genome complexity.

5 Exploring molecular diagnostics for NTM infections: beyond pathogen identification to advances in DST

Merely identifying the pathogen is insufficient for clinical satisfaction. Once the species of mycobacteria in a clinical sample is identified, the crucial next step is to determine its drug susceptibility to devise an appropriate treatment strategy. Inherent resistance, characteristic of certain NTM species, guides initial antibiotic choices, whereas acquired resistance, emerging during treatment, necessitates ongoing monitoring and potentially adjusting therapeutic strategies. While studies on MTB have laid the foundation for understanding antibiotic resistance mechanisms, the genetic and phenotypic diversity among NTM species necessitates targeted research to uncover specific resistance patterns and develop effective treatments. Increasing reports indicate that antibiotics previously effective, including macrolides, fluoroquinolones, and aminoglycosides, are now facing resistance, an issue particularly prevalent and posing a higher risk in slow-growing NTM, exacerbated by the extensive use of these antibiotics in both clinical and animal husbandry. Settings, leading to increased selection pressure for resistant strains (128, 129).

Managing NTM infections is notably challenging, largely owing to two main factors. First, the relationship between *in vitro* DST outcomes and clinical success is not consistently direct. This inconsistency can be attributed to factors such as the complex biology of NTM species and variability in patient responses to treatment. For example, DST results for *M. abscessus* and *Mycobacterium simiae* (*M. simiae*) have a weak correlation with clinical efficacy, in contrast to species such as *M. kansasii*, *M. marinum*, and *M. fortuitum*, where there is a stronger alignment with treatment success (130, 131). This variation in correlation may be due to differences in the mechanisms of antibiotic resistance and pathogenicity among these species, affecting the predictability of treatment outcomes based on DST alone. Secondly, clinical case management ranges from no treatment to requiring a multidrug regimen. The decision to opt for no treatment, monitor, or pursue aggressive multidrug regimens is guided by a comprehensive evaluation of the specific NTM species involved, infection severity, and individual patient health considerations, highlighting the need for a personalized approach to treatment. Thus, DST, aligned with Clinical and Laboratory Standards Institute (CLSI) guidelines, is critical for crafting optimal treatment strategies. Adhering to CLSI guidelines, which are regularly updated to reflect emerging data on NTM behavior and antibiotic resistance, ensures that DST is applied effectively to inform treatment strategies, underscoring the dynamic nature of NTM management. Identification of clinically significant antibiotic resistance genes (ARGs) enables the prediction of pathogen resistance.

The CLSI guidelines were revised and expanded in 2018, establishing broth microdilution as the most common laboratory method for DST to determine the minimum inhibitory concentration (MIC) (132). Traditional culture-dependent approaches for DST, prevalent in clinical microbiology laboratories, suffer from drawbacks including lengthy processing times, bias towards certain microbial

communities, and contamination risks from overgrowth. Recent studies have elucidated the intrinsic and acquired resistance mechanisms of mycobacteria to classes of antibiotics including macrolides, aminoglycosides, oxazolidinones (such as linezolid), riminophenazines (like clofazimine), and di-substituted diazepanes (such as bedaquiline), paving the way for the application of molecular diagnostic techniques in DST (133). Notably, molecular diagnostics can also detect resistance heterogeneity, identifying resistant subpopulations within drug-sensitive mycobacterial communities (134, 135). The Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca) aggregates well-documented, peer-reviewed resistance determinants alongside their corresponding antibiotics. Utilizing CARD facilitates the identification of genes and mutations associated with NTM resistance, enabling comparison of these sequences against WGS and NGS data. Regrettably, no existing database perfectly correlates NTM resistance predictions from WGS data with DST outcomes, reinforcing the need for refined bioinformatic tools and databases. Table 2 showcases a meticulously compiled inventory of NTM-relevant mutations and genes, including their reference data, sourced from the CARD (136–150). Table 3 outlines the comparison of DST and WGS in antibiotic susceptibility identification for NTM.

Given the predominance of macrolide antibiotics as the first-line treatment for most NTM infections, the detection of resistance to these drugs is particularly critical. Macrolide resistance mechanisms involve the methylation of 23S rRNA by the erythromycin ribosome methylation (*erm*) gene, preventing macrolide binding to its ribosomal target. Additionally, mutations in the 23S rRNA itself can render macrolides ineffective, a scenario relatively common in mycobacteria due to their limited number of rRNA operons. A single mutation in any of these operons can significantly alter the ribosomal structure, inhibiting macrolide binding (151–153). Rifampin remains among the preferred treatments for most NTM infections, generally linked to mutations in its target, the *rpoB* gene, which encodes the β-subunit of RNA polymerase. Studies suggested rifampin preferentially inhibits one of the rpoB promoters (Promoter I), leading to increased expression from the second promoter (Promoter II) and promoting the growth of resistant strains (154). Recent research indicates additional mechanisms might be involved. There are reported cases of rifampin resistance in both MAC and *M. kansasii* (155, 156). *M. abscessus* resistance constitutes a formidable clinical hurdle, as evidenced by multiple DST studies demonstrating resistance across several antibiotic classes, including macrolides, quinolones, and aminoglycosides. Notably, intrinsic high-level resistance has been predominantly associated with established mutations in the *embB* gene, conferring resistance to ethambutol, and the *gyrA* gene, conferring resistance to fluoroquinolones (157). Additionally, intrinsic resistance to rifampin in *M. abscessus* is due to mutations in the *rpoB* gene and the presence of the *MAB_0591* gene, which is known to contribute to rifampin resistance processes (158). Another mechanism of intrinsic resistance is the overexpression of efflux pumps, contributing to resistance against bedaquiline and clofazimine (159). Chen et al. (160) conducted WGS on clofazimine-resistant *M. abscessus* strains, revealing several significant mutations in the *MAB_2299c*, *MAB_1483*, and *MAB_0540* genes, which were found to play crucial roles in conferring drug resistance. The identification of the *erm41* gene and its pivotal role in macrolide resistance has facilitated the critical differentiation of three distinct species within the MABC: *M. abscessus* subsp. abscessus (MABSa), *M. abscessus* subsp. massiliense (MABSm), and *M. abscessus* subsp. bolletii (MABSb). Prior to this discovery, the American Thoracic Society/Infectious Disease Society of America (ATS/IDSA) guidelines on NTM infections did not reflect the central influence of the *erm41* gene on macrolide susceptibility. This oversight has significant clinical implications, as the majority of MABC isolates exhibit a functional erm41 gene, correlating with poor treatment outcomes in patients (161). In MABSa, a T/C polymorphism at position 28 of the *erm41* gene determines inducible macrolide resistance (28 T) or susceptibility (T28C). While most MABSa and MABSb strains exhibit inducible macrolide resistance, those with the T28C substitution are sensitive due to the loss of *erm41* functionality. Conversely, MABSm contains a large deletion in the *erm41* gene, resulting in nonfunctional *erm41* and macrolide susceptibility (162, 163). The commercialized GenoType NTM-DR kits (Hain Life science, Germany) enables rapid identification of *M. abscessus* subspecies and concurrent detection of resistance to macrolides and aminoglycosides. Studies have demonstrated the test's high sensitivity in detecting acquired resistance, underscoring its utility in the molecular diagnostics landscape for NTM infections (164, 165).

The adoption of WGS has markedly advanced our comprehension of NTM antibiotic resistance, thus improving treatment strategies. In a significant validation of WGS's precision, Wetzstein et al. (166) demonstrated complete concordance between WGS forecasting of *M. abscessus* resistance to macrolides and aminoglycosides and results from both GenoType NTM-DR kits and phenotypic DST. Lipworth et al. (167) leveraged sequencing data from *M. abscessus* to identify novel mutations in the *erm* gene and *rrs* gene linked to macrolide resistance, mutations not covered by traditional genotyping, raising the possibility of false-negative outcomes. Yoshida et al. (168) developed a WGS-based diagnostic approach, incorporating DNA chromatography and PCR, to distinguish macrolide-resistant and macrolide-sensitive *M. abscessus* subspecies, including *M. massiliense*, *M. abscessus*, and *M. bolletii*, achieving a 99.7% concordance with conventional DST findings. With a promising start, Realegeno et al. (169) have formulated a WGS assay aimed at accurately predicting *M. abscessus* resistance to clarithromycin and amikacin, achieving an impressive 100% accuracy when compared to phenotypic results. Yet, it should be noted that before wide clinical application of such a WGS-based prediction method, extensive validation against an array of drugs and broader NTM species is imperative. NGS, encompassing both mNGS and tNGS, is increasingly valued for its potential in detecting drug resistance genes in NTM. tNGS, in particular, sequences specific genes or genomic regions, offering higher specificity in detecting target genes compared to mNGS (126). Due to the highly conserved DNA sequences within the Mycobacterium and limited nucleotide sequence data, mNGS often fails to accurately identify species within the Mycobacterium or detect multiple drug-resistant mutations (170). tNGS combines multiplex PCR amplification with sequencing to rapidly capture diverse drug resistance gene sequences, thus advancing the molecular diagnosis of NTM resistance (124, 125).

6 Clinical applications of molecular diagnostic techniques in the diagnosis of NTM

In contemporary clinical practice, advances in molecular biology have revolutionized the diagnosis of NTM infections. These

10.3389/fpubh.2024.1410672

TABLE 2 Mutated genes associated with NTM and their mutation details retrieved from the CARD database.

NTM species	AMR gene family	CARD short name	Drug class	Resistance mechanism	References
M. intracellulare	23S rRNA with mutation conferring resistance to azithromycin	*Mint_23S_AZM*	Macrolide	antibiotic target alteration	(134)
	23S rRNA with mutation conferring resistance to clarithromycin	*Mint_23S_CLR*	Macrolide	antibiotic target alteration	(134)
M. avium	23S rRNA with mutation conferring resistance to clarithromycin	*Mavi_23S_CLR*	Macrolide	antibiotic target alteration	(135)
	Fluoroquinolone resistant *gyrA*	*Mavi_gyrA_FLO*	Fluoroquinolone	antibiotic target alteration	(136)
M. kansasii	23S rRNA with mutation conferring resistance to clarithromycin	*Mkan_23S_CLR*	Macrolide	antibiotic target alteration	(137)
M. abscessus	Antibiotic resistant ATP synthase	*Mabs_atpE_BDQ*	Diarylquinoline	Antibiotic target alteration	(138)
	16S rRNA mutation conferring resistance to kanamycin	*Mabs_16S_KAN*	Aminoglycoside	Antibiotic target alteration	(139)
	16S rRNA mutation conferring resistance to neomycin	*Mabs_16S_NEO*	Aminoglycoside	Antibiotic target alteration	(140)
	16S rRNA mutation conferring resistance to gentamicin	*Mabs_16S_GEN*	Aminoglycoside	Antibiotic target alteration	(139)
	16S rRNA mutation conferring resistance to tobramycin	*Mabs_16S_TOB*	Aminoglycoside	Antibiotic target alteration	(139)
	23S rRNA with mutation conferring resistance to clarithromycin	*Mabs_23S_CLR*	Macrolide	Antibiotic target alteration	(141, 142)
	16S rRNA mutation conferring resistance to amikacin	*Mabs_16S_AMK*	Aminoglycoside	Antibiotic target alteration	(141, 142)
M. chelonae	16S rRNA mutation conferring resistance to kanamycin A	*Mche_16S_KAN*	Aminoglycoside	Antibiotic target alteration	(140)
	16S rRNA mutation conferring resistance to neomycin	*Mche_16S_NEO*	Aminoglycoside	Antibiotic target alteration	(140)
	16S rRNA mutation conferring resistance to gentamicin C	*Mche_16S_GENC*	Aminoglycoside	Antibiotic target alteration	(140)
	16S rRNA mutation conferring resistance to tobramycin	*Mche_16S_TOB*	Aminoglycoside	Antibiotic target alteration	(140)
	23S rRNA with mutation conferring resistance to clarithromycin	*Mche_23S_CLR*	Macrolide	Antibiotic target alteration	(143)
	16S rRNA mutation conferring resistance to amikacin	*Mche_16S_AMK*	Aminoglycoside	Antibiotic target alteration	(140)
	23S rRNA with mutation conferring resistance to clarithromycin	*Msme_23S_CLR*	Macrolide	Antibiotic target alteration	(144)
M. smegmatis	23S rRNA with mutation conferring resistance to clarithromycin	*Msme_23S_CLR*	Macrolide	Antibiotic target alteration	(144)
	16 s rRNA with mutation conferring resistance to aminoglycoside antibiotics	*Msme_16rrsA_HGM*	Aminoglycoside	Antibiotic target alteration	(145)
	16 s rRNA with mutation conferring resistance to aminoglycoside antibiotics	*Msme_16rrsB_HGM*	Aminoglycoside	Antibiotic target alteration	(145)
	Antibiotic resistant *ndh*	*Msme_ndh_INH*	Isoniazid-like	Antibiotic target alteration	(146)
	16 s rRNA with mutation conferring resistance to aminoglycoside antibiotics	*Msme_16rrsB_STR*	Aminoglycoside	Antibiotic target alteration	(147)
	16 s rRNA with mutation conferring resistance to peptide antibiotics	*Msme_16rrsB_VIO*	Peptide	Antibiotic target alteration	(148)
	16 s rRNA with mutation conferring resistance to aminoglycoside antibiotics	*Msme_16rrsB_KAN*	Aminoglycoside	Antibiotic target alteration	(148)
	16 s rRNA with mutation conferring resistance to aminoglycoside antibiotics	*Msme_16rrsA_KAN*	Aminoglycoside	Antibiotic target alteration	(148)
	16 s rRNA with mutation conferring resistance to aminoglycoside antibiotics	*Msme_16rrsA_NEO*	Aminoglycoside	Antibiotic target alteration	(148)
	16 s rRNA with mutation conferring resistance to aminoglycoside antibiotics	*Msme_16rrsB_NEO*	Aminoglycoside	Antibiotic target alteration	(148)

AMR, antimicrobial resistance; CARD, the comprehensive antibiotic resistance database.

TABLE 3 Comparison of DST and WGS in antibiotic susceptibility identification for NTM.

Methods	Advantages	Limitations	Optimizing strategy
DST	• Reliable and valid. • Cost-effective and straightforward. • Provides clear results and direct therapeutic guidance based on guidelines.	• Extended duration, complex calibration, and tough quality control cause variable outcomes. • Manual handing causes inconsistencies. • Weak link between lab tests and clinical efficacy, with poor detail on drug resistance profiles.	• Develop new culturing techniques and media to: • Broaden testing scope. • Reduce turnaround times. • mplement standardized testing and quality control protocols.
WGS	• High-resolution methods identify more mutations quickly. • Eliminate need for existing resistance databases. • Enhance data sharing and comparative analysis.	• Lack of precise antimicrobial resistance databases reduces accuracy. • High costs and technical requirements demand advanced bioinformatics and tools. • DNA must be extracted from positive cultures,not directly from primary samples.	• Establish extensive databases and sophisticated tools. • Adopt standardized testing and quality control protocols. • Consolidate databases to improve precision in medical research.

DST, drug sensitivity testing; WGS, whole genome sequencing.

methodologies offer rapid, accurate, and comprehensive detection capabilities for NTM species identification and genotyping, along with critical insights into antimicrobial resistance and susceptibility. The systematic standardization, which ensures consistency and reliability across different laboratories, and a modular approach that allows for the tailored combination of diagnostic tests, together with rigorous quality control measures, significantly enhance the efficacy and reliability of clinical diagnostics and thereby inform more precise therapeutic strategies.

Numerous molecular techniques are applicable for species identification and genotyping of NTM, each offering distinct advantages and specific applications in clinical settings. Techniques such as MLVA, MIRU-VNTR, Rep-PCR, RFLP, PFGE, RAPD, AFLP, and LSP are primarily employed for NTM subspecies in clinical diagnostics. Conversely, methods such as hybridization probes, WGS, and NGS provide significant advantages in identifying drug resistance genes. This capability is particularly critical in anticipating NTM drug resistance, especially in the absence of available DST. Figure 1 depicts the application and workflow of molecular diagnostic techniques in NTM species and subspecies identification.

7 Revolutionizing NTM classification and phylogeny with molecular diagnostic

The delineation and phylogenetic affiliations of NTM remain critical challenges due to their diverse genetic backgrounds and widespread environmental presence, which complicate clinical identification and treatment. Conventional methodologies, primarily relying on phenotypic characteristics and limited genetic markers, often lead to inconsistent and conflicting taxonomic identifications when different tools analyze the same environmental samples. The NTM taxonomy is dynamic, with phylogenetic updates substantially influencing the terminology across various fields. Advancements in molecular diagnostics, particularly through 16S rRNA gene amplification and sequencing, have unveiled extensive microbial diversity and intricate interrelations (171, 172). Nonetheless, exclusive reliance on 16S rRNA gene-based taxonomy presents significant shortcomings, such as inadequate resolution at and below the genus level, primer mismatches, and the potential distortion of phylogenetic relationships owing to chimeric sequence formation during PCR, which may impact the integrity of phylogenetic trees (173, 174). Thus, there's a necessity for genomic data, beyond the 16S rRNA gene sequences, for accurate species classification and matching (175). Analyses of metagenomes and the construction of phylogenetic trees using single-copy, vertically transmitted protein sequences provide enhanced resolution compared to those derived from a single phylogenetic marker gene, such as 16S rRNA. These approaches are increasingly recommended for taxonomic reference (176, 177). The advent of the molecular diagnostics era, marked by the application of technologies such as NGS, including WGS and mNGS, has not only clarified NTM genomes but also provided a more accurate and comprehensive understanding of their phylogenetic relationships, thereby overcoming the limitations of previous methodologies. Genomic data enables the construction of more robust, comprehensive phylogenetic trees with higher resolution. A comprehensive review of the literature was undertaken, focusing on instances where molecular methodologies have been utilized to construct phylogenetic and evolutionary analyses of NTM, as detailed in Table 4 (178–185).

8 Integration of molecular diagnostic technologies with novel pathogen identification techniques

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers significant advantages for biomolecular analysis (186). It has proven effective for identifying pathogenic microorganisms and their resistance patterns, demonstrating specificity and sensitivity superior to Sanger sequencing, with detection limits on par with next-generation sequencing (187–191). While the identification of bacterial isolates, including NTM, is well established using MALDI-TOF MS, the technology is optimized for pure cultures. Challenges arise with mixed Mycobacterium cultures, where molecular methods are necessary to identify multiple NTM species in respiratory specimens. Despite its utility, differentiation of closely related species,

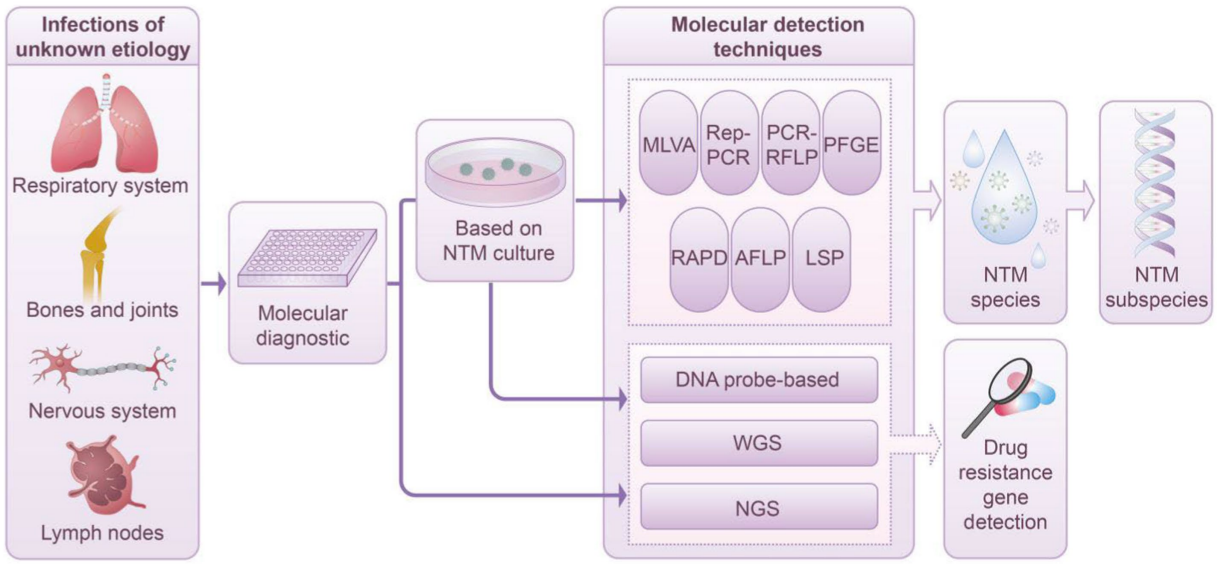

FIGURE 1
Diagnostic workflow based on molecular technology in NTM infection management. This schematic depicts the integration of advanced molecular techniques within the clinical framework for the diagnosis of NTM infections. Techniques such as MLVA, Rep-PCR, PCR-RFLP, PFGE, RAPD, AFLP, LSP, and DNA probe-based techniques, along with WGS and NGS, facilitate the detailed characterization of NTM species and their subspecies. The process entails isolating and culturing a singular NTM colony from a clinical specimen, followed by its purification. Notably, cutting-edge methodologies such as DNA probe-based techniques, WGS, and NGS enable direct application to clinical samples, thereby circumventing the conventional culture step. Upon completing the identification of NTM species and subspecies, tools such as DNA probe-based techniques, WGS, and NGS are employed to comprehensively evaluate NTM resistance genes. This approach significantly enriches our understanding of the mechanisms underlying microbial resistance.

such as subspecies of *M. abscessus*, poses difficulties. Literature reports occasional misidentifications by MALDI-TOF MS, primarily among closely related species or those within the same mycobacterial complex (192). Although some studies have explored subspecies identification through protein peak analysis via MALDI-TOF MS, a consensus on the optimal approach remains elusive (193–195). Recent years have seen the adoption of advanced data analysis techniques, including machine learning, which extend beyond simple species identification to provide more comprehensive insights (196). While MALDI-TOF MS has demonstrated efficacy in DST, it has yet to gain global consensus (197). In summary, MALDI-TOF MS offers rapid, sensitive, accurate, and cost-effective detection with high resolution and minimal sample requirements, enabling precise identification of NTM. Nevertheless, for complex NTM infections, integration with newer molecular diagnostic technologies is essential for accurate identification.

High-performance liquid chromatography (HPLC), developed in the mid-1960s within the chemical sciences, has been extensively applied across inorganic, organic, and biochemical disciplines (198). In the realm of NTM species identification, HPLC facilitates the analysis of mycobacterial components like mycolic acids, offering a faster alternative to traditional, time-intensive identification methods (199). While HPLC enhances species-level differentiation over biochemical tests, its effectiveness is limited by its inability to consistently discern clinically significant NTM species (200). Moreover, the proliferation of similar HPLC chromatograms among various species has diminished its utility as a standalone diagnostic tool (201). Consequently, for robust and accurate NTM species identification and typing, HPLC should be integrated with other molecular diagnostic techniques.

9 Limits and future challenges in molecular diagnostic for NTM identification

Despite the benefits of molecular diagnostics for NTM infection identification, the literature identifies key limitations and challenges. The lack of standardized protocols results in operational and data processing variability, impacting comparability and reproducibility across labs. Additionally, the necessity for high-quality samples and the variability in sample sources complicate standardizing pretreatment procedures, affecting detection accuracy and reliability. Furthermore, the elevated costs and complexity of certain molecular methods limit their broad clinical adoption, underscoring the need for more affordable and straightforward technologies to enhance their practicality and acceptance in clinical settings.

In upcoming research, molecular diagnostics will focus on key areas. Microfluidic technologies, known for their high throughput, sensitivity, and cost-effectiveness, are expected to revolutionize NTM detection by enabling automated sample processing and analysis. Additionally, integrating artificial intelligence (AI) and machine learning (ML) shows promise in extracting insights from extensive molecular data, aiding in precise diagnostic and treatment decisions. Nucleic acid mass spectrometry (NAMS) has gained prominence, with clinical laboratories increasingly adopting these techniques, poised to become standard in routine lab settings, offering potential for NTM infection detection advancements (202). Advances in genome editing technologies, like CRISPR/Cas9, offer opportunities for precise NTM genome editing, enhancing understanding of pathogenic mechanisms, drug responses, and resistance (203). These technological

TABLE 4 Published data on studies involving molecular diagnostic techniques in constructing phylogenetic and evolutionary of NTM.

Author	Year	Bacteria	Methodology	Outcome	References
Fedrizzi et al.	2017	41 NTM type strains	NGS	Found clearly distinct evolutionary pathways for slow and rapidly growing mycobacteria in agreement with the pre-NGS era phylogeny.	(176)
Tortoli et al.	2017	144 NTM type strains	NGS	Refined the phylogeny of NTM by expanding the analysis to 88 strains	(177)
Gupta et al.	2018	150 NTM type strains	WGS	Phylogenetic trees were constructed for 150 members of the genus mycobacterium	(178)
Chew et al.	2021	*M. abscessus*	WGS	The risk of patient-to-patient transmission appears to be largely limited to the vulnerable CF population, indicating infection from environmental sources remains more common than human-to-human transmission	(179)
Das et al.	2018	*M. marinum*	WGS	*M. marinum* should be divided into two different clusters, the "M"- and the "Aronson"-type	(180)
Uchiya et al.	2017	*M. avium*	WGS	MAH had the highest degree of sequence variability among the subspecies, and MAH strains isolated in Japan and those isolated abroad possessed distinct phylogenetic features.	(181)
Stinear et al.	2008	*M. marinum*	WGS	Comparisons with the more distantly related *M. avium* subspecies paratuberculosis and *M. smegmatis* reveal how an ancestral generalist mycobacterium evolved into MTB and *M. marinum*	(182)
Qi et al.	2009	*M. ulcerans*	NGS	Offered significant insight into the evolution of *M. ulcerans* and provide a comprehensive report on genetic diversity within a highly clonal *M. ulcerans* population from a Buruli ulcer endemic region.	(183)
Matsumoto et al.	2019	175 NTM type strains	NGS	Expanded the available genomic data to 175 NTM species and redefined their subgenus classification	(82)

NGS, next-generation sequencing; WGS, whole genome sequencing; CF, cystic fibrosis; MAH, *Mycobacterium avium* subsp. hominissuis; MTB, *Mycobacterium tuberculosis*.

advancements will deepen comprehension of NTM diseases and drive diagnostic and therapeutic methodologies forward.

10 Conclusion

Molecular diagnostics, notably through WGS and NGS, have revolutionized NTM infection research, providing efficient, precise tools for species identification and drug sensitivity prediction. These methods have exceeded traditional limitations, offering fresh insights into NTM treatment approaches. Yet, challenges remain in studying less common NTM species, highlighting the need for technique refinement and a deeper understanding of NTM diversity.

In summary, the significant influence of molecular diagnostics on NTM identification is undeniable. As research progresses and technological refinement continues, there exists a potential for pioneering discoveries that will elucidate the complex biology of NTM and enable the development of more precise and effective diagnostic and therapeutic strategies for conditions related to NTM.

Author contributions

HZ: Funding acquisition, Methodology, Resources, Writing – original draft. TM: Conceptualization, Writing – original draft. DL: Supervision, Validation, Writing – review & editing. MX: Conceptualization, Writing – original draft. YA: Investigation, Writing – review & editing. LL: Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by the key research and development project of the Sichuan Science and Technology Department (No. 2022YFS0268).

Acknowledgments

The authors thank all staff involved in the implementation of this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 28 June 2024
DOI 10.3389/fvets.2024.1409694

OPEN ACCESS

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RECEIVED 30 March 2024
ACCEPTED 18 June 2024
PUBLISHED 28 June 2024

CITATION
Ottardi M, Lechner I, Wang J, Schmitt S, Schneeberger M, Schmid RM, Stephan R and Meylan M (2024) Seroprevalence of *Mycobacterium avium* subsp. *paratuberculosis* in Swiss dairy herds and risk factors for a positive herd status and within-herd prevalence.
Front. Vet. Sci. 11:1409694.
doi: 10.3389/fvets.2024.1409694

Seroprevalence of *Mycobacterium avium* subsp. *paratuberculosis* in Swiss dairy herds and risk factors for a positive herd status and within-herd prevalence

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Introduction: Bovine paratuberculosis (PTB) is a chronic enteric disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Control of PTB is important given its negative economic consequences and the potential zoonotic role of MAP in Crohn's disease in humans.

Methods: To determine the seroprevalence of MAP in Swiss dairy herds and to identify risk factors associated with seropositive herd status and high within-herd seroprevalence, 10,063 serum samples collected from cattle over 12 months of age in 171 Swiss dairy farms were analyzed using a commercial ELISA test. Eight herds were excluded due to non-interpretable ELISA results. Risk factors associated with seropositive herd status and high within-herd seroprevalence were investigated with regression models using results from a questionnaire on management practices possibly associated with the introduction or spread of MAP in the remaining 163 herds. Univariable logistic regression was performed, carrying forward for multivariable regression analysis when $p < 0.2$.

Results: The calculated between-herd true seroprevalence was 3.6% (95% CI, 0.96–8.4%). Due to the low within-herd seroprevalence, it was not possible to calculate the true seroprevalence at animal level; the apparent within-herd seroprevalence ranged from 2.3 to 5.5% with a median of 3.6% in nine positive farms. Herd size ($p = 0.037$) and the common grazing of lactating cows with cows from other herds ($p = 0.014$) were associated with seropositive herd status, while heifers sharing alpine pasture with dairy cattle from other herds were associated with a decreased probability of the herd to test seropositive ($p = 0.042$). Reliable identification of significant risk factors associated with MAP spread and high seroprevalence of PTB within seropositive herds was not possible due to low observed seroprevalence within herds and low sensitivity of the ELISA test.

Discussion: These results highlight the limitation of serology for MAP diagnosis in small herds with low infection prevalence.

KEYWORDS

paratuberculosis, dairy, serology, ELISA, low seroprevalence, small herds, risk factors, Switzerland

1 Introduction

Paratuberculosis (PTB) is a fatal chronic intestinal infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), affecting primarily domestic and wild ruminants such as red deer, but also other wildlife including rabbits, foxes and badgers (1–4). Also known as Johne's disease (JD), PTB is characterized by a long incubation period during which animals may remain subclinically infected for years (5). The cardinal clinical signs, weight loss and watery diarrhea, develop at a late stage of infection; finally, the disease leads to the death of affected animals. Young animals under 6 months of age are at the highest risk of becoming infected in herds with PTB (6). First reported in Germany at the end of the 19th century, PTB is now widely distributed throughout the world and is considered a significant disease due to its multiple negative impacts on economy and animal welfare (5, 7–9).

Intensive animal purchase, especially from multiple herds of origin, is a major risk factor associated with MAP introduction into cattle herds (10–13). Contact with feces of infected animals is also a potential risk factor for the introduction of the disease, and herd size is a recognized risk factor associated with a positive herd status as well (14, 15). Within-herd MAP transmission is primarily associated with contact of calves with adult cows' feces (16), whereby transmission to young calves occurs mainly by ingestion of milk or feed contaminated with fecal material from infected animals (5). Beside indirect contamination of colostrum and milk through fecal material, MAP may also be directly secreted by the mammary gland of infected cows, especially in late stages of the disease (17–19). Intrauterine infection has also been described, particularly in cows with clinical JD (20). Management of the calving area and contact of the newborn calves with their dams are important points in the control of within-herd transmission (21, 22).

Mycobacterium avium subsp. *paratuberculosis* has been suspected to represent a zoonotic risk as a trigger agent for Crohn's Disease (CD), a human chronic inflammatory bowel disease (23–29). Since MAP can be secreted directly into the milk of infected cows (18), a possible transmission way to humans could be through consumption of milk and dairy products (30). In many countries, cow milk is generally pasteurized prior to consumption, however, MAP has been shown to be able of surviving commercial pasteurization (31, 32). Insufficient understanding of MAP's zoonotic potential and of its role in the development of human disease hampers a well-founded evaluation of the magnitude of its impact on public health (33, 34).

Economic losses due to PTB are mainly attributable to reduced milk production, animal replacement costs and decreased slaughter value (9, 35–39). In Switzerland, an annual economic loss of approximately CHF 4.6 millions due to reduced milk production was recently calculated for a population of 559,900 dairy cows (40). The fact that PTB can be spread through the movement of subclinically infected animals that contaminate their new environment, e.g., after purchase in a new herd, is considered to have contributed to the dissemination of JD (e.g., infected animals of continental European origin suspected of being a source of JD in Ireland), and has therefore serious implications for animal trade (9, 13, 41–44).

Although numerous countries have formal, mostly voluntary PTB control programs based on testing and culling strategies (9, 45, 46), these programs mostly have limited success due to the long incubation period and the low efficiency of diagnostic methods to identify infected animals during the subclinical phase of the disease (41, 47, 48). The sensitivity (Se) of different diagnostic tests varies considerably, ranging, e.g., between 7 and 94% for serum Enzyme-Linked Immunosorbent Assays (ELISA), 29 to 61% for milk ELISA, and 23 and 74% for fecal culture (FC), however, variations in study design and used diagnostic methods make comparison of different results almost impossible (49, 50). The delay of several years between infection time and detectable MAP shedding or immune reaction means that the same test is likely to perform better when used for animals with clinical JD than for animals in the subclinical stage of infection, e.g., the Se of a serum ELISA was found to be 87% in cows with clinical PTB and only 15% in subclinically infected animals (51–53). Likewise, serum ELISA showed a higher Se in animals with a heavy bacterial load (>50 colony forming units (CFU) per tube; Se = 75%) compared to low shedders (<10 CFU/tube; Se = 15%) (54). While FC allows for detecting infected animals earlier in the course of disease than other methods, it requires long incubation times (up to 16 weeks) until a definitive result is available (5, 52), and it can only detect animals excreting the organism, resulting in false-negative outcome in infected animals that have not yet started to shed the bacterium (55). Furthermore, the intensive laboratory work and expertise required for culturing are associated with high costs. In contrast, PCR methods are faster, easier and cheaper, but it has been shown that they may have a distinctly lower Se for MAP detection than FC (56). Therefore, despite the disadvantage of limited Se, quick and unexpensive serological tests are still commonly used to determine the prevalence of MAP infection at the herd level (49).

Numerous studies have been conducted worldwide to estimate PTB prevalence in cattle (9, 15, 46, 57, 58). More than 20% of the herds were considered to be infected with MAP in approximately half of 48 countries around the world for which data were available (9). The disease has been recently classified as enzootic in 27 European countries (including Switzerland), i.e., "countries where the disease was present and for which all periods of absence were shorter than 2 years" (46). Prevalence estimates have generally been higher for dairy cattle than for beef cattle (7). In North America, a study conducted in the 17 main dairy U.S. states in 2007 indicated that 68.1% herds had at least one cow that tested positive on FC (59). In Europe, the apparent between-herd seroprevalence in cattle was reported to range from 38 to 68% (60). However, available prevalence studies are heterogeneous and it is difficult to compare their results due to differences in sampling design, diagnostic strategies and case definitions (41). In many regions of the world, the prevalence of PTB is still completely unknown (45).

In Switzerland, where PTB is a notifiable disease, approximately 60 bovine cases have been reported yearly in the last 3 years (61), however, given the long subclinical phase of infection and unspecific clinical signs, PTB may be more common but affected animals are culled without diagnosis confirmation. Sparse prevalence data at the herd and at the animal level are also available from earlier studies (62–66), but current information about the actual prevalence of PTB in Swiss dairy herds is not available, despite the importance of the dairy industry in Switzerland. The aim of the present study was to determine the between-herd and the within-herd seroprevalence of PTB in a representative subset of Swiss dairy herds. A serum ELISA method was used to allow for inclusion of large numbers of animals and herds. In addition, information about the herds was collected in

order to investigate risk factors associated with positive herd status and seroprevalence within positive herds.

2 Materials and methods

2.1 Study design

Farms were recruited for an observational seroprevalence study and risk factor analysis on PTB in a representative subset of the Swiss dairy population.

2.2 Sample size

The numbers of herds and animals to be sampled was calculated in a two-step procedure using Epitools® (67), based on the data available from previous studies in Switzerland (62, 64, 68, 69). First, the sample size to assess the within-herd prevalence was determined, whereby a minimum Se and Sp of ≥95% for herd status determination was set as target performance. A within-herd prevalence of 20% and a median of 30 animals ≥1 year old were assumed for the calculation. The diagnostic test Se and Sp were 58.2 and 99.0%, based on test characteristics of an approved ELISA tests (IDEXX Paratuberculosis Screening Ab Test, IDEXX Montpellier SAS, Montpellier, France) by the Friedrich-Loeffler-Institute (70). Second, the sample size to assess the between-herd prevalence was determined, assuming a between-herd prevalence of 20% and using the target Se and Sp of the within-herd calculations. This resulted in a target sample size of 300 herds (confidence level of 95%, precision of 5%).

2.3 Study population: farms and animals

"Herd size" was defined as the number of dairy cows (in lactation and dry cows), and "number of animals" was used to describe the total of animals tested including heifers from the age of 12 months until 2 years throughout the manuscript. Inclusion criteria for the farms were a herd size of at least 25 dairy cows (in order to achieve the calculated 30 tested animals when including cows and heifers over the age of 12 months) and membership in at least one of the main Swiss dairy breeding associations: Holstein, Braunvieh and Swissherdbook, which represented about 88% of the registered dairy cows in Switzerland in 2022 (71–73). Heifers between the age of 12 and 24 months were included in the study population based on the results of a previous Swiss study (66) in which 3.9% of the heifers in this age category had been found to be shedding MAP (positive fecal culture), in 6 out of 13 participating herds. Participants for the study were recruited via an email sent by the breeding associations. The email comprised a document describing the project and a link for online enrolment through a short questionnaire (SurveyMonkey platform, Momentive Global Inc., Delaware St, San Mateo, USA). The questionnaire, available in German, French and Italian, consisted of 19 questions, the first five ones about the farmers' contact information and identification number of the farm (in the Swiss animal movement database, TVD), and the remaining ones about farm management and animals (type of production, average number of animals, main breed, use of alpine pastures and whether young animals went to heifer raising facilities). The responding farmers gave written informed consent for access to individual cow data (cow identification, date of birth, last date of calving) by the study team as well as the farm's membership number in one (or more) breeding association(s) to obtain information on milk yield and quality as well as on reproduction parameters. The email was sent to approximately 11,000 possible participants fulfilling the inclusion criteria in all regions of Switzerland. The survey remained open for 3 months from July 19, 2021, to September 21, 2021. The study was approved by the competent Veterinary authorities (animal experimentation authorization number BE 32–2021).

2.4 Sampling

Each farmer who had enrolled in the study via the online questionnaire was contacted by telephone to confirm his/her willingness to participate in the study and to set up an appointment for a farm visit. Farm visits took place between November 2021 and October 2022, each farm was visited once. Farmers who reported sending (mostly young) animals to alpine pastures during the summer were visited during the winter period to gain access to all testable animals. All cows (in lactation and dry) and heifers older than 12 months and less than 2 years were included in the study. Blood samples were collected from the coccygeal vein using Serum Monovettes® (9 mL Z, SARSTEDT AG & Co. KG, Nümbrecht, Germany). The samples were transported in a container refrigerated at 1°C to the Vetsuisse Faculty Bern, where they were centrifuged (2,123 *g*, 10 min) and the serum separated within maximal 12 h of collection. The serum was transferred to 2 mL Micro Tubes® (SARSTEDT AG & Co. KG, Nümbrecht, Germany) and frozen at −20°C. The frozen serum samples were then transported at monthly intervals to the Institute of Veterinary Bacteriology of the University of Zurich for analysis.

2.5 Serological analysis

The frozen sera were thawed and tested with the commercial ELISA ID Screen Paratuberculosis Indirect Screening Test [IDvet, Grabels, France; Se 58.2%, Sp 99.3% (70)] according to the manufacturer's instructions. The sera were tested in duplicate, and the optical densities (OD) were recorded at 450 nm. Results were interpreted according to the manufacturer's instructions (greater than or equal to 70% was considered positive, less than or equal to 60% was considered negative, and greater than 60% and less than 70% was considered doubtful).

2.6 Farm questionnaire

The questionnaire to assess risk factors for MAP introduction and spread in the farms was developed in Microsoft Access 2016 (Microsoft Corporation, Redmond, Washington, USA). Questions were based on questionnaires used in previous studies (13, 16, 48, 66, 74–78), the questionnaire was validated with 3 farmers prior to use in the frame of the study. The questionnaire was filled at the end of the farm visit, after blood sampling, during a personal interview with the farm manager. If

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the manager was not present at that time, the interview was done later by phone. Information on farm demographics and management was gathered with the questionnaire (Table 1), as well as information about 10 major themes including calving area, housing, feeding management, manure and slurry management, use of pasture and/or alpine pasture, animal trade, knowledge of PTB, previous cases of the disease, and a last section about hygiene (Tables 2–5). The animals on the farm were assigned to 6 categories according to their age (neonates in the first 2–3 weeks of life (housed in individual hutches or small groups), older (pre-weaned) calves until weaning, post-weaned calves until 1 year of age, bred heifers from approximately 1 year until calving, lactating cows, and dry cows) according to the handbook for the Risk Assessments and Management Plans for Johne's Disease of the National Johne's Working Group (41). The questionnaire sections on housing, feeding, pasture and alpine pasture were recorded for each age group separately.

2.7 Data management and statistical analyses

Data from the risk assessment questionnaire were recorded in a Microsoft Excel file (Microsoft Corporation, Redmond, Washington, USA). Additional farm production data made available by the breeding associations included farm annual production reports, individual monthly milk weighing and standard lactation data, as well as insemination and calving dates of the cows. Individual cow data were provided directly by the participants as Microsoft Excel files or obtained through the Swiss online counter for agriculture (79). The participating herds were classified as positive or negative for PTB based on ELISA results. Since farms of different sizes were included, the cut-off for a seropositive herd status was adjusted so that both Se and Sp remained above 95%: one serologically positive animal defined the herd as seropositive if herd size (number of animals aged 2 years or more years, i.e., cows in lactation and dry cows) was 38 or less, two positives for 39–81 animals, and three for 85–138 animals. The number of reactors was assessed with the Herd Sensitivity/Specificity Calculator of Scotland's Rural College [SRUC (80)]. In addition to the apparent seroprevalence, the true seroprevalence accounting for the diagnostic test characteristics was calculated, for within-herd prevalence using the diagnostic test characteristics and for between-herd prevalence using the median Se and S*p* values over all herds; these calculations were performed with Epitools® (67).

A risk factor analysis was performed to identify possible associations of management factors with a seropositive herd status as well as with the within-herd prevalence in infected herds. For the identification of possible associations of management factors with a seropositive herd status, factors possibly associated with MAP introduction in dairy herds were assessed (Table 2). For the risk factor analysis considering the within-herd prevalence, risk factors influencing MAP spread within infected herds were considered (Tables 3–5). Since youngstock of some herds were raised in external rearing farms, and because of differences due to barn types (free stall vs. tie stall), some answers were missing for questions about young animals and calving management. To minimize missing data, some of the original questionnaire variables and their categories were grouped for analyses (see Supplementary Tables S1, S2). The data for

TABLE 1 Summary of farm demographics, herds characteristics and previous experience with paratuberculosis (PTB) for the 163 Swiss dairy farms participating in the study, classified as seropositive ($n = 9$) or seronegative ($n = 154$) for PTB based on serum ELISA results.

	Total	Seropositive farms	Seronegative farms
Farm size (hectares)			
Median	37	45	35
IQR[1]	25.0–50.0	30.0–75.0	25.0–50.0
Range (minimum-maximum)	11–160	25–85	11–160
Herd size (all cows ≥2 years)			
Median	42	50	41
IQR[1]	32–57	41–55	32–57
Range	16–138	36–138	16–133
Average milk yield (liters/cow/year)			
Median	7,800	8,600	7,800
IQR[1]	7,000–9,000	7,500–9,000	7,000–9,000
Range	5,000–17,000	5,500–17,000	5,000–15,000

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Farm PTB-status	163 (100)	9 (5.5)	154 (94.5)
Agricultural zone			
Midland zone	86 (52.7)	4 (44.4)	82 (53.2)
Hill zone	25 (15.3)	3 (33.3)	22 (14.3)
Mountain zones[2]	52 (32.0)	2 (22.2)	50 (32.5)
Stall system			
Free stall	140 (85.9)	8 (88.9)	132 (85.7)
Tie-stall	23 (14.1)	1 (11.1)	22 (14.3)
Production system			
Conventional[3]	91 (55.8)	6 (66.7)	85 (55.2)
Label[4]	55 (33.7)	3 (33.3)	52 (33.8)
Organic[5]	17 (10.5)	0 (0)	17 (11.0)
Reproduction management			
Breading bull on farm	37 (22.7)	1 (11.1)	36 (23.4)
Artificial insemination only	126 (77.3)	8 (88.9)	118 (76.6)
Main cattle breed			
Holstein/Red Holstein	78 (47.9)	6 (66.7)	72 (46.8)
Brown Swiss	49 (30.0)	1 (11.1)	48 (31.2)
Swiss Fleckvieh	15 (9.2)	0 (0)	15 (9.7)
Other breeds[6]	21 (12.9)	2 (22.2)	19 (12.3)

(Continued)

TABLE 1 (Continued)

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Seasonal calving[7]			
Yes	11 (6.7)	2 (22.2)	9 (5.8)
No	152 (93.3)	7 (77.8)	145 (94.2)
Previous PTB cases in the herd			
Yes	13 (8.0)	5 (55.6)	8 (5.2)
No	122 (74.8)	3 (33.3)	119 (77.3)
Unknown	28 (17.2)	1 (11.1)	27 (17.6)
Origin of the sick animals			
Animal(s) raised on farm	11 (84.6)	4 (80.0)	7 (87.5)
Purchased animal(s)	2 (15.4)	1 (20.0)	1 (12.5)

[1]Interquartile range: first quartile (25th percentile) – third quartile (75th percentile).
[2]Including all four mountain zones defined in the Swiss agricultural system: map.geo.admin.ch.
[3]As described in: Ökologischer Leistungsnachweis (admin.ch).
[4]As described in: www.ipsuisse.ch.
[5]As described in: www.biosuisse.ch.
[6]Including Jersey, Montbéliarde, Kiwi-Cross, Normande, Simmentaler and crossbreeds.
[7]Seasonal calving: systems focused on having most cows calve over a short period (about 12 weeks) starting in late winter so that the period of maximum feed demand coincides with the period of peak pasture growth rates around mid-spring.

risk factors possibly associated with a seropositive herd status were analyzed in two categories (Supplementary Table S1).

Statistical analyses were performed using the R software, version 4.2.3 (R Core Team 2023). Before conducting the main analyses, the number of cows, which was used as a proxy for herd size, was logarithmized. Moreover, pairwise correlation tests to the response variables were applied to prevent multicollinearity and model overfitting. Spearman's rank correlation matrix was performed and any pair of variables with a correlation coefficient (|r|) greater than 0.8 was considered highly correlated (81). However, no variable was collinear, and, therefore, all response variables were retained for further analysis. For both datasets, an univariable logistic regression was performed, carrying forward for multivariable regression analysis when $p < 0.2$. Regarding the multivariable regression analysis, variables were removed if the constructed model exhibited the lowest Akaike information criterion (AIC) after using the stepwise elimination method, aiming at achieving the most parsimonious model. The multivariable regression models set a significance cut-off of $p < 0.05$. For the first model analysis, the response variables were farms seropositive for PTB according to the definition given above. After selecting the most parsimonious model in the multivariable model, interactions among the response variables were assessed. For the second model, the response variable was the prevalence of PTB seropositivity (number of seropositive cases/number of tested animals *100) in each herd. Lastly, all resulting models were inspected visually for homoscedasticity and normality.

3 Results

3.1 Study population: farms and animals

Serum samples were collected in 171 dairy farms distributed over all 26 cantons of Switzerland, except for Appenzell Inner-Rhodes, Basel-City, Geneva and Obwalden. Median herd size was 42 dairy cows aged 2 years or more (IQR: 32–57), with Holstein being the predominant breed (in 47.9% of all farms, 66.7% of positive farms and 54.9% of seropositive animals). A total of 10,063 animals (7,943 cows, 2,091 heifers and 29 bulls) were tested for antibodies against MAP, the number of animals tested per herd ranged from 22 to 199, with a median of 8 heifers aged between 1 and 2 years and 42 cows aged 2 years or more. A case of PTB was reported to have previously occurred in 13 farms (8%). Participation to the study was on a voluntary basis and recruiting 300 farms with a minimum of 30 animals for testing turned out to be impossible. Therefore, slightly smaller farms with a minimum of 25 animals were also included in the study. In three cases, however, less than 25 animals were eventually available for testing (once 22 and twice 23, respectively) because the heifers were not on site at the time of sampling. Thus, the target sample size of 300 herds could not be achieved. However, since several very large (under Swiss dairying conditions) farms were sampled, the average herd-level Se was higher than the initially set target of 95%. Consequently, a reasonable CI for the final between-herd prevalence calculations could be achieved despite the lower than expected number of participating herds.

3.2 Serological analyses

Of 10,063 serum samples that were tested for antibodies against MAP, 51 (46 cows and 5 heifers) were positive, 10,000 (7,886 cows, 2,085 heifers and 29 bulls) were negative, and 12 (11 cows and one heifer) were doubtful. Eight farms (with 453 animals, 347 cows and 106 heifers) had to be excluded from further analyses because one (or several) ELISA result in the herd was doubtful and the farm could not be reliably classified as negative or positive. The ELISA results of the remaining 163 herds are presented in Table 6. Fifty-one of the 9′610 remaining samples (0.53%) tested positive, most of them were cows ($n = 46/7{,}596$, 0.61%) but five heifers (between the age of one and 2 years) were also antibody positive ($n = 5/1{,}985$, 0.25%); all tested bulls were seronegative. Information on farm demographics and herd characteristics is shown in Table 1.

At the herd level, nine of the remaining 163 farms (5.5%) fulfilled the criteria for seropositivity based on the number of animals in the herd as described above. The geographical distribution of the 163 farms included in the study is shown in Figure 1. In these nine seropositive farms, 25 animals (of the 9,610 tested, 0.26%) were seropositive; all 25 seropositive animals were cows aged between 3.0 and 9.5 years, with a median of age of 5.0 years. A single cow was seropositive in one herd, two cows in six herds, and more than two (four and eight, respectively) in two farms. Herd size of the nine seropositive herds ranged from 36 to 138, with a median of 50 cows aged 2 years or more. Holstein was the predominant breed among the seropositive animals (14/25, 56%), and Jersey the second most predominant (9/25, 36%); the herd with the highest number of seropositive animals (eight among the 146 tested) was composed exclusively of Jerseys. The between-herd true seroprevalence calculated using a Se of 0.99 and a Sp of 0.98 was 3.6% (95% CI, 0.96–8.4%). The within-herd apparent seroprevalence in the nine seropositive herds ranged from 2.3 to 5.5% with a median of 3.6%, and the calculated within-herd true seroprevalence ranged from 2.8 to 8.3%, with a median of 4.9%. Due to the low apparent animal seroprevalence, it was not possible to calculate the true prevalence at animal level. Seropositive animals were present in 23 further farms that did not fulfill the criteria

TABLE 2 Herd level occurrence of characteristics and management practices included in the analyses of risk factors potentially associated with a seropositive herd status for paratuberculosis in Swiss dairy herds, for all participating farms (total, $n = 163$) and for serologically positive ($n = 9$) and negative ($n = 154$) herds separately.

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Type(s) of production on the farm			
Exclusively dairy	121 (74.2)	6 (66.7)	115 (74.7)
Dairy and fattening of calves born on the farm as veal and/or beef	35 (21.5)	2 (22.2)	33 (21.4)
Dairy and fattening of purchased veal calves	4 (2.5)	0 (0)	4 (2.6)
Dairy and fattening of purchased beef calves	3 (1.8)	1 (11.1)	2 (1.3)
Presence of a bull in the herd			
No bull or one raised on the farm	115 (70.6)	7 (77.8)	108 (70.0)
New bull purchased <1x per year	23 (14.1)	0 (0)	23 (15.0)
New bull purchased every year	25 (15.3)	2 (22.2)	23 (15.0)
Origin of the farm's water supply			
Communal water source only	95 (58.3)	5 (55.6)	90 (58.4)
Private well (with or without additional communal water source)	68 (41.7)	4 (44.4)	64 (41.6)
Contact with animals from other herds during the grazing period[8]			
Calves (pre-weaned and post-weaned)			
No[9]	163 (100)	9 (100)	154 (100)
Heifers			
No[9]	103 (63.2)	6 (66.7)	97 (63.0)
With youngstock[10]	59 (36.2)	3 (33.3)	56 (36.4)
With adult cattle[11]	1 (0.6)	0 (0)	1 (0.6)
Lactating cows			
No[9]	160 (98.0)	8 (88.9)	152 (98.7)
With adult cattle[11]	3 (2.0)	1 (11.1)	2 (1.3)
Dry cows			
No[9]	157 (96.3)	8 (88.9)	149 (96.8)
With adult cattle[11]	6 (3.7)	1 (11.1)	5 (3.2)
Shared alpine pasture with animals from other herds[12]			
Pre-weaned calves			
No[13]	161 (98.7)	9 (100)	152 (98.7)
With adult cattle[11]	2 (1.3)	0 (0)	2 (1.3)
Post-weaned calves			
No[13]	140 (85.9)	9 (100)	131 (85.1)
With youngstock[10]	16 (9.8)	0 (0)	16 (10.3)
With adult cattle[11]	7 (4.3)	0 (0)	7 (4.6)
Heifers			
No[13]	28 (17.2)	4 (44.4)	24 (15.6)
With youngstock[10]	110 (67.5)	5 (55.6)	105 (68.2)
With adult cattle[11]	25 (15.3)	0 (0)	25 (16.2)

(Continued)

TABLE 2 (Continued)

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Lactating cows			
No[13]	134 (82.2)	8 (88.9)	126 (81.8)
Yes[14]	29 (17.8)	1 (11.1)	28 (18.2)
Dry cows			
No[13]	141 (86.5)	9 (100)	132 (85.7)
Yes[14]	22 (13.5)	0 (0)	22 (14.3)
Participation to cattle shows (≥once a year)			
Yes	62 (38.0)	3 (33.3)	59 (38.3)
No	101 (62.0)	6 (66.7)	95 (61.7)
Purchase of breeding animals			
No	109 (66.9)	6 (66.7)	103 (66.9)
Only as young animals[10]	5 (3.0)	0 (0)	5 (3.2)
At least one adult animal[15] per year	49 (30.1)	3 (33.3)	46 (29.9)
Number of source farms for purchase[16]			
No purchase	77 (47.2)	2 (22.2)	75 (48.7)
From one farm	31 (19.0)	2 (22.2)	29 (18.8)
From > one farm	55 (33.8)	5 (55.6)	50 (32.5)
Information request about the source farm[17]			
Yes	9 (5.5)	1 (11.1)	8 (5.2)
No	154 (94.5)	8 (88.9)	146 (94.8)
Heifers raised on a rearing farm[18]			
Yes	56 (34.4)	2 (22.2)	54 (35.1)
No	107 (65.6)	7 (77.8)	100 (64.9)

[8]Direct contact of the different animal categories with animals from other farms during the grazing period.
[9]No direct contact with animals from other farms or only via adjacent pastures.
[10]Including calves and heifers.
[11]Including lactating and/or dry cows.
[12]Shared alpine summer pasture with animals from other farms for the different animal categories.
[13]Animals of the farm do not go to summer pasture or they do not share it with animals from other farms.
[14]Including any combination of calves, heifers and cows.
[15]Including cows or bulls.
[16]Number of farms from which cattle was purchased in the last year.
[17]Request for information on potential diseases in the farm of origin by the farmer prior to purchase.
[18]Young animals raised in an external rearing farm.

for seropositivity. Herd size of these farms ranged from 29 to 90 cows, with a median herd size of 54 cows. In 20 out of these 23 herds (87%), only one animal was seropositive, of which five were heifers; two cows were found to be seropositive in the remaining three herds.

3.3 Risk factor analysis on herd serostatus for paratuberculosis

The results of the univariable regression analysis regarding herd serostatus (positive or negative for PTB) are shown in Supplementary Table S1. Four variables exhibiting *p*-values below 0.2, were selected for inclusion in the subsequent multivariable regression analysis, i.e., "contact of lactating cows with animals from other herds during grazing," "heifers sharing alpine pasture with animals from other herds," "source of animals for purchase" and "herd size." The results of the multivariable regression incorporating this variable set reaffirmed the significance of three of these four variables as explanatory factors for herd serostatus with *p*-values lower than 0.05, i.e., "contact of lactating cows with animals from other herds during grazing," "heifers sharing alpine pasture with animals from other herds," and "herd size" (Table 7A). The variables "herd size" and "contact of lactating cows with animals from other herds during grazing" emerged as significant positive contributors to the herd's PTB serostatus, while "heifers sharing alpine pasture with animals from other herds "was identified as a significant negative contributor.

3.4 Risk factor analysis for within-herd seroprevalence

Three variables had *p*-values below 0.2 in the univariable regression analysis regarding within-herd seroprevalence, including "milk feeding," "heifers sharing alpine pasture with other animal

TABLE 3 Herd level occurrence of characteristics and management practices potentially associated with contact between animals of different age categories within a farm included in the analysis of risk factors potentially associated with the prevalence level within herds seropositive for paratuberculosis in Swiss dairy herds, for all participating farms (total, $n = 163$) and for serologically positive ($n = 9$) and negative ($n = 154$) herds separately.

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Presence of other ruminants on the farm[19]			
Yes	55 (33.7)	3 (33.3)	52 (33.8)
No	108 (66.3)	6 (66.7)	102 (66.2)
Presence of other food producing animals (i.e., pigs)[20] on the farm			
No	129 (79.1)	8 (88.9)	121 (78.6)
<20 pigs	12 (7.4)	0 (0)	12 (7.8)
≥20 pigs	22 (13.5)	1 (11.1)	21 (13.6)
Calving management			
Contact with other cows during calving			
No, only individual calving[21]	75 (46.0)	4 (44.5)	71 (46.1)
Low contact intensity[22]	26 (16.0)	1 (11.0)	25 (16.2)
Intensive contact (group calving box)[23]	62 (38.0)	4 (44.5)	58 (37.7)
Bedding management for calving[24]			
Change of bedding after each calving	28 (17.2)	3 (33.3)	25 (16.2)
Pen or stall freshly bedded before each calving[25]	122 (74.8)	4 (44.5)	118 (76.6)
Same bedding used several times[26]	13 (8.0)	2 (22.2)	11 (7.2)
Gloves for the care of newborn calves			
Yes	6 (3.7)	0 (0)	6 (3.9)
No	157 (96.3)	9 (100)	148 (96.1)
Use of the calving pen as sick pen			
Never	20 (12.3)	1 (11.1)	19 (12.3)
Rarely	34 (20.6)	2 (22.2)	32 (20.8)
Often	16 (9.8)	0 (0)	16 (10.4)
Always	71 (43.5)	4 (44.5)	67 (43.5)
N/A[27]	22 (13.5)	2 (22.2)	20 (13.0)
Colostrum from the dam's udder[28]			
Yes	80 (49.0)	2 (22.2)	78 (50.6)
No	83 (51.0)	7 (77.8)	76 (49.3)
Housing of the young animals			
Neonates (first 2–3 weeks of life)			
Individual hutch[29]	92 (56.4)	5 (55.5)	87 (56.5)
Group pen	71 (43.6)	4 (44.5)	67 (43.5)
Pre-weaned calves			
Individual hutch[29]	3 (1.8)	0 (0)	3 (1.9)
Group pen	156 (95.7)	9 (100)	147 (95.5)
Not on the farm (rearing farm)[18]	4 (2.5)	0 (0)	4 (2.6)
Post-weaned calves			
Group pen	137 (84.0)	7 (77.8)	130 (84.4)
Not on the farm (rearing farm)[18]	26 (16.0)	2 (22.2)	24 (15.6)

(Continued)

TABLE 3 (Continued)

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Risk of fecal contamination from the cows to the calves' environment			
Neonates (first 2–3 weeks of life)			
No[30]	4 (2.5)	1 (11.1)	3 (1.9)
Low[31]	151 (92.6)	7 (77.8)	144 (93.5)
Moderate[32]	0 (0)	0 (0)	0 (0)
High[33]	8 (4.9)	1 (11.1)	7 (4.6)
Pre-weaned calves			
No[30]	6 (3.7)	1 (11.1)	5 (3.3)
Low[31]	147 (90.2)	7 (77.8)	140 (90.9)
Moderate[32]	3 (1.8)	0 (0)	3 (1.9)
High[33]	7 (4.3)	1 (11.1)	6 (3.9)
Post-weaned calves			
No[30]	14 (8.6)	0 (0)	14 (9.1)
Low[31]	32 (19.6)	2 (22.2)	30 (19.5)
Moderate[32]	111 (68.1)	7 (77.8)	104 (67.5)
High[33]	6 (3.7)	0 (0)	6 (3.9)
Shared alpine pastures with other animal categories of the farm			
Heifers			
No	134 (82.2)	9 (100)	125 (81.2)
Yes[34]	29 (17.8)	0 (0)	29 (18.8)
Lactating cows			
No[35]	156 (95.7)	9 (100)	147 (95.5)
Yes[34]	7 (4.3)	0 (0)	7 (4.5)
Dry cows			
No[35]	148 (90.8)	9 (100)	139 (90.3)
Yes[34]	15 (9.8)	0 (0)	15 (9.7)

[19]Including goats, sheep, new world camelids, water buffalo, deer.
[20]No food producing animals other than pigs were present in the participating farms.
[21]Including individual calving pen or tie stall where the neighboring cows are removed prior to a calving.
[22]Calving on pasture (extensive) or tie stall where the neighboring cows are not removed before calving (maximal two cows).
[23]Calving in the barn or group calving pen (up to 20 cows).
[24]Cleaning of calving pen and for tie stall change of bedding before the calving.
[25]Bedding not removed, new clean material added.
[26]In case of tie stall: not freshly bedded before calving.
[27]N/A: not applicable (no calving pen).
[28]Colostrum intake through direct nursing from the dam.
[29]Single housing in box or igloo, only one calf per box or igloo with visual contact with the other calves but no physical contact.
[30]No contamination possible on the farm, or the calves are not on the farm (rearing farm).
[31]Low risk: indirect contamination through the use of same cleaning or feeding equipment and boots both for the calves' and the cows' area.
[32]Moderate risk: calves are grazed on the same pastures or alp pasture with cows.
[33]High risk: calves are housed with the cows or can come in contact with cows or cow manure in their housing.
[34]All combinations of animals of different ages from the same farm.
[35]Not with young animals but possible contact between lactating and dry cows.

categories of the herd" and "occurrence of diseases in cows" (Supplementary Table S2). These variables were subsequently retained for inclusion in the multivariable regression model. Two of them, "milk feeding" and "occurrence of diseases in cows" remained significantly, albeit negatively associated with PTB seroprevalence (Table 7B).

3.5 Farmer's knowledge about paratuberculosis

Questionnaire results on farmers' knowledge about PTB are shown in Table 8. Only 24.5% of the participants had been aware of the existence of PTB prior to the study, the rest of them reported not

TABLE 4 Herd level hygiene management practices potentially associated with the spread of *M. avium* subsp. *paratuberculosis* within a farm included in the analysis of risk factors potentially associated with the prevalence level within herds seropositive for paratuberculosis in Swiss dairy herds, for all participating farms (total, *n* = 163) and for serologically positive (*n* = 9) and negative (*n* = 154) herds separately.

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Frequency of manure removal			
Neonates (first 2–3 weeks of life)			
1-2x/day	24 (14.7)	0 (0)	24 (15.6)
<1x/day	139 (85.7)	9 (100)	130 (84.4)
Pre-weaned calves			
>3x/day	3 (1.8)	1 (11.1)	2 (1.3)
1-2x/day	26 (16.0)	0 (0)	26 (16.9)
<1x/day	130 (79.7)	8 (88.9)	122 (79.2)
N/A[36]	4 (2.5)	0 (0)	4 (2.6)
Post-weaned calves			
>3x/day	12 (7.4)	1 (11.1)	11 (7.1)
1-2x/day	48 (29.4)	2 (22.2)	46 (29.9)
<1x/day	77 (47.2)	4 (44.5)	73 (47.4)
N/A[36]	26 (16.0)	2 (22.2)	24 (15.6)
Heifers			
>3x/day	22 (13.5)	1 (11.1)	21 (13.6)
1-2x/day	45 (27.6)	3 (33.3)	42 (27.3)
<1x/day	48 (29.4)	3 (33.3)	45 (29.2)
N/A[36]	48 (29.4)	2 (22.3)	46 (29.9)
Lactating cows			
>3x/day	109 (66.9)	5 (55.6)	104 (67.5)
1-2x/day	42 (25.8)	4 (44.4)	38 (24.7)
<1x/day	12 (7.3)	0 (0)	12 (7.8)
Dry cows			
>3x/day	67 (41.1)	3 (33.3)	64 (41.6)
1-2x/day	59 (36.2)	4 (44.5)	55 (35.7)
Not daily	37 (22.7)	2 (22.2)	35 (22.7)
Cleaning management in the calf area			
Neonates (first 2–3 weeks of life)			
Regular[37] disinfection[38]	16 (9.8)	1 (11.1)	15 (9.7)
1-4x/year disinfection[39]	79 (48.5)	5 (55.6)	74 (48.1)
Cleaning/washing[40]	11 (6.7)	0 (0)	11 (7.1)
No disinfection or washing	57 (35.0)	3 (33.3)	54 (35.1)
Pre-weaned calves			
Regular[37] disinfection[38]	9 (5.5)	1 (11.1)	8 (5.2)
Disinfection 1-4x/year[39]	26 (16.0)	1 (11.1)	25 (16.2)
Cleaning/washing[40]	2 (1.2)	0 (0)	2 (1.3)
No disinfection or washing	126 (77.3)	7 (77.8)	119 (77.3)
Post-weaned calves			
Regular[37] disinfection[38]	1 (0.6)	0 (0)	1 (0.6)
Disinfection 1-4x/year [39]	9 (5.5)	0 (0)	9 (5.8)
Cleaning/washing[40]	1 (0.6)	0 (0)	1 (0.6)
No disinfection or washing	152 (93.3)	9 (100)	143 (93.0)

(Continued)

TABLE 4 (Continued)

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Runoff from the manure pile[41]			
Yes	114 (70.0)	8 (88.9)	106 (68.8)
No	49 (30.0)	1 (11.1)	48 (31.2)
Use of manure equipment for other tasks[42]			
No	158 (96.9)	9 (100)	149 (96.8)
After cleaning also for feed[43]	5 (3.1)	0 (0)	5 (3.2)
Occurrence of diseases at the herd level at the time of farm visit[44]			
Calves			
Yes	119 (73.0)	1 (11.1)	118 (76.6)
No	40 (24.5)	8 (88.9)	32 (20.8)
N/A[36]	4 (2.5)	0 (0)	4 (2.6)
Heifers			
Yes	105 (64.4)	6 (66.7)	99 (64.3)
No	6 (3.7)	1 (11.1)	5 (3.2)
N/A[36]	52 (31.9)	2 (22.2)	50 (32.5)
Cows[45]			
Yes	49 (30.0)	0 (0)	49 (31.8)
No	114 (70.0)	9 (100)	105 (68.2)

[36]N/A: not applicable (youngstock in external rearing farm).
[37] Regular: after each group.
[38] Disinfection with a product active against mycobacteria (i.e., Neopredisan® or Noviralx3®; other disinfectants without efficacy against mycobacteria used in six farms are listed under "cleaning/washing").
[39]Disinfection of the area > 1x year.
[40]Area cleaned with hot water, high pressure or disinfected with a disinfectant without efficacy against mycobacteria.
[41]Contamination of the environment around the manure pile.
[42]Vehicles and equipment for loading and transporting manure also used for other purposes (feed transport).
[43]Cleaned with water.
[44]Presence of diseases considered as a problem at the herd level at the time of the visit, including: for calves digestive, respiratory, or umbilical diseases; for heifers digestive diseases and fertility problems; for cows digestive diseases, fertility problems and poor udder health; or any other problems mentioned by the herd managers for every category.
[45]Including lactating and dry cows.

having heard about the disease before and not being aware of clinical signs related to it. Among the herd managers who indicated having some knowledge of the disease, most recognized the two cardinal symptoms of chronic diarrhea and emaciation, while only few of them were aware of a reduction in milk yield and fertility. Only 35% of the farmers aware of the disease knew at least one infection pathway, whereby oral infection via feces and intrauterine infection were mentioned most often.

4 Discussion

The main aim of the present study was to estimate the national PTB herd and animal level prevalences in a large sample of Swiss dairy cattle herds, and to evaluate risk factors potentially associated with a positive herd serostatus and with the within-herd seroprevalence in positive herds. The apparent between-herd seroprevalence of 5.5% detected in this survey is distinctly lower than the apparent between-herd seroprevalence reported in previous studies, e.g., in a review of several studies conducted in European countries (38–68%) (60), or in a study conducted in Northern Italy (48% in Lombardy and 65% in Veneto (82)). At the individual animal level, the apparent seroprevalence of 0.53% is also lower than reported among adult cows in other Europeans countries, e.g., 2.6% in Lombardy and 4.0% in Veneto (82) or from 4.4 to 10.3% in Hungary (83). The median apparent within-herd seroprevalence was 3.6% in the nine seropositive herds. Previous prevalence records of MAP infection in Swiss dairy cattle are limited to few earlier studies (62–66). However, none of these studies provided reliable data for Switzerland due to small sample sizes or lack of representativity of the study population. Because participation in the present study was voluntary, a selection bias with farmers knowing or suspecting the presence of PTB in their herd and choosing not to participate for fear of possible consequences cannot be excluded. Although positive serological results had no regulatory consequences for the participating farms (this was confirmed in the mail sent to recruit participants), some farmers may have preferred not to be involved in a study on a notifiable disease. The recruitment of participating herds turned out to be more challenging than expected, although it was done in collaboration with the main breeding associations, with written confirmation by their boards that they recommended participation to this study on a disease of importance to the dairy industry. This eventually led to a number of

TABLE 5 Herd level feeding management practices potentially associated with the spread of *M. avium* subsp. *paratuberculosis* within a farm included in the analysis of risk factors potentially associated with the prevalence level within herds seropositive for paratuberculosis in Swiss dairy herds, for all participating farms (total, *n* = 163) and for serologically positive (*n* = 9) and negative (*n* = 154) herds separately.

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Feeding management			
Milk feeding[46]			
Powdered milk	13 (8.0)	2 (22.2)	11 (7.1)
Fresh milk (with/without milk powder)[47]	150 (92.0)	7 (77.8)	143 (92.9)
Leftovers from the cows' feed given to post-weaned calves			
Yes	15 (9.2)	0 (0)	15 (9.7)
No	148 (90.8)	9 (100)	139 (90.3)
Possible fecal contamination of calf feed[48]			
Yes	10 (6.1)	0 (0)	10 (6.5)
No	153 (93.9)	9 (100)	144 (93.5)
Leftovers from the cows' feed given to heifers			
Yes	34 (20.9)	2 (22.2)	32 (20.8)
No	129 (79.1)	7 (77.8)	122 (79.2)
Possible water contamination[49] from cows to calves (all calf categories)			
Yes	3 (1.9)	0 (0)	3 (1.9)
No	160 (98.1)	9 (100)	151 (98.1)
Use of manure and/or slurry for fertilization			
For crop land and/or hay meadows	5 (3.0)	0 (0)	5 (3.2)
For cow and heifer pastures	44 (27.0)	2 (22.2)	42 (27.3)
For pastures, including calf pastures	114 (70.0)	7 (77.8)	107 (69.5)

[46]Feeding of the calves after colostrum administration.
[47]Milk or a mix of milk and milk powder.
[48]Possible contamination of calves' feed with cow manure in the feed storage or on the feeding place.
[49]Possible fecal contamination of the calves' water with cow manure.

TABLE 6 Results of the serum ELISA test (ID Screen® Paratuberculosis Indirect Screening Test, IDvet, Grabels, France) for the detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in 9,610 bovines aged 1 year or more in 163 Swiss dairy herds.

Results	*n* cows	*n* heifers >1 year	*n* bulls	Total (%)
Positive	46	5	0	51 (0.53)
Negative	7,550	1,980	29	9,559 (99.47)
Total	7,596	1,985	29	9,610 (100)

participating herds lower than the calculated sample size. This suggests that (mandatory) sampling in a randomly selected subpopulation of dairy farms would be necessary for a really representative study on PTB in Switzerland. The exclusion of small farms in order to mitigate limited test accuracy through recruitment of herds with 25 cows or more may have further complicated the acquisition of participants for the study, given the relatively small average size of Swiss dairy herds. However, 40 animals or more were tested in most participating herds (121 out of 163, 74.2%). The farms included in the study were distributed in all agricultural zones of Switzerland (midland, hill, and mountain zones I-IV). This division into agricultural zones aims at representing the degree of difficulty in production and living conditions to be taken into consideration in the application of the Agriculture Act, e.g., some subsidies provided to the farmers depend on the zone where their farm is located. In the study population, a larger proportion of the farms (52.7%) was located in the midland zone compared to the proportion in the entire Swiss dairy population (32.5% of the herds). In contrast, the four mountain zones together were less represented in the study population in comparison with the general Swiss dairy farm distribution (32% vs. 52.2%). The distribution in the hill zone was the same in both populations (15.3%). This observation is likely due to the selection of farms with a minimal herd size of 25 dairy cows, as farms in the mountain zones tend to be smaller compared to the farms in the midland zone, with an average of 30.5 cows and 18.5 cows, respectively, in 2022 (84).

The observed seroprevalence is surprisingly low, despite the large number of samples collected in more than 160 herds. The serological analyses were conducted in duplicate in a certified laboratory (the Swiss reference laboratory for mycobacterial diseases in animals) with a commercial ELISA-kit, thus an unnoticed technical problem appears

unplausible. One concern about using ELISA methods for the detection of positive herds is the possible misclassification of farms due to the low Se of serological tests in early stages of the infection, as antibodies are rarely present in sufficient quantities to be detected during the subclinical phase of PTB (21, 51). Based on the characteristics of the test used (Se = 58.2%, Sp = 99.3%), false negative results must be expected at the individual animal level. Given the contagious nature of PTB, it is not surprising that the odds of herd seropositivity should increase with herd size, which has been observed in numerous studies worldwide (14, 15, 66, 74, 85), including serological studies (12, 21, 22, 86). However, the higher probability of finding positive animals when more animals are tested in larger herds than in small herds must also be taken into consideration. This is the reason why we chose to sample and test all animals (from the age of 1 year old) in the participating herds, in order to increase the likelihood of detecting seropositive herds. Indeed, 146 (120 cows and 26 heifers) and 171 (138 cows and 33 heifers) animals were tested in the two farms with 8 and 4 seropositive animals, respectively, between 41 and 76 animals in the herds with two seropositive animals, and 36 in the smallest farm that was classified as positive with one seropositive cow. In order not to mitigate better Se with decreased Sp at the herd level due to the large number of tested animals, a cutoff (minimal number of seropositive animals needed to classify a herd as positive depending on the number of tested animals) was determined for each herd individually to ensure a Se and a Sp of 95% or more at herd level. Herd size, calculated based on the number of cows only in the models in order to include the herds with heifer rearing outside of the farm, remained significantly associated with a positive herd status, indicating that other factors than the number of tested animals alone contribute to the risk of being PTB positive in larger herds. These other factors may include, e.g., more animal purchase, larger groups of animals, or less control of management practices on large farms with hired employees than in small family farms.

The predominant breed in the participating farms was Holstein with 47.9% of the herds. These were mostly large herds, with a median herd size of 46.5 cows. In contrast, Swiss dairy farms are generally smaller with an average herd size of 23 cows (84). Although the Jersey breed has been reported to be more susceptible for PTB than other dairy breeds (85, 87, 88), breed was not identified as a risk factor in our analyses. The Jersey breed is not very common in Switzerland, as only 5,108 cows were registered with the breeding association in 2022 (71), which corresponded to 0.9% of the total dairy cow population in Switzerland (84). In our study, Jersey cattle was predominant in four herds (2.4%), one of which was composed exclusively of Jerseys. Nevertheless, 36% (9/25) of the seropositive animals in the positive herds were Jerseys, and the herd with the highest number of seropositive animals (8/146, 5.5%) was the one with Jersey cattle only. However, given the low number of observations, these results should not be overinterpreted.

Despite the precautions taken to minimize erroneous classification of herds, we know of at least one herd that was likely falsely classified as seronegative in the study as one cow (tested negative by ELISA in November 2021) later exhibited clinical signs of JD; according to the owner, the diagnosis was confirmed by examination of a fecal sample (method unknown). Thirty-five animals (24 cows and 11 heifers) had been tested in the herd, the positive cow was, to the best of our

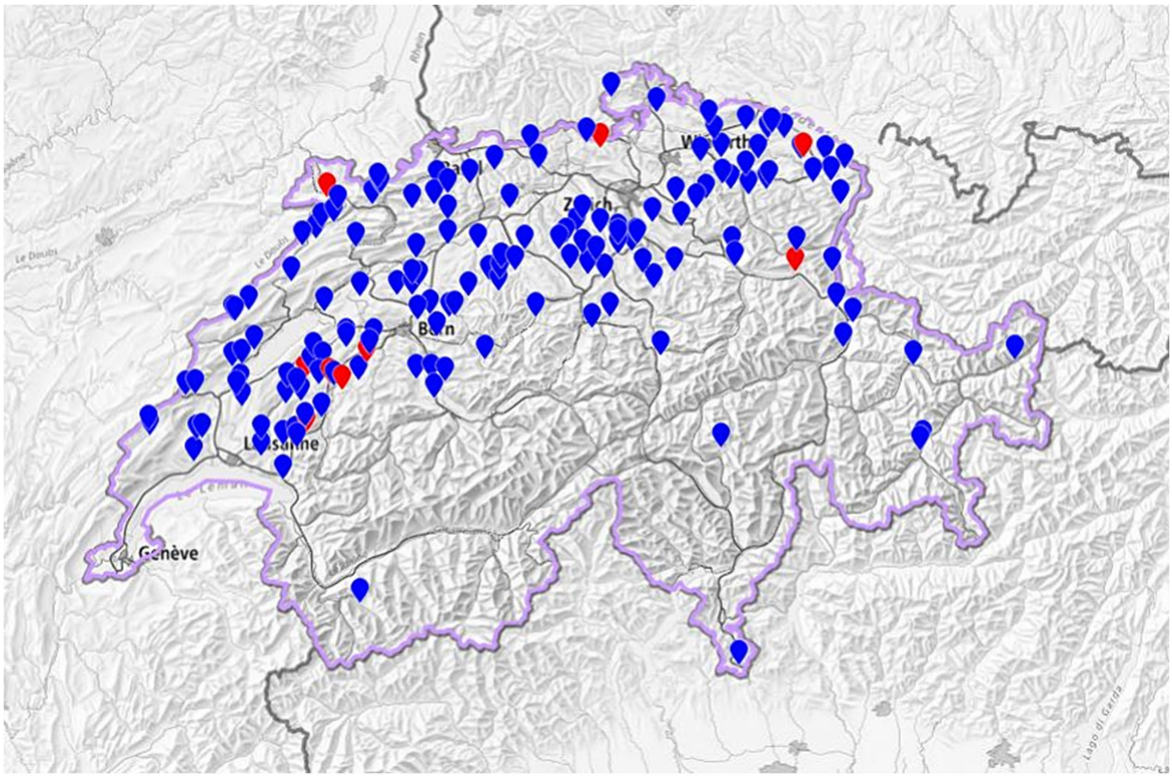

FIGURE 1
Geographical distribution of the 163 Swiss dairy farms included in the study [9 seropositive (red dots) and 154 seronegative (blue dots) herds]. Based on study data mapped in the Swiss Federal Geoportal https://map.geo.admin.ch/.

TABLE 7 Results of the multivariable logistic regression models assessing possible associations (A) between risk factors and herd serostatus (seropositive or seronegative for paratuberculosis) and (B) between risk factors and within-herd seroprevalence in 163 Swiss dairy herds (9 seropositive, 154 seronegative); statistically significant associations ($p \leq 0.05$) are indicated in bold.

(A) Multivariable logistic regression model regarding risk factors for a positive herd serostatus					
Variable	Coefficient	OR[50]	SE[51]	95% CI[52]	*p* value
Intercept	−9.09	0	3.62	<0.000	0.012
Contact of lactating cows with animals from other herds during grazing Yes (vs. no)	3.6	36.8	1.47	2.05–658.54	**0.014**
Heifers sharing alpine pasture with animals from other herds Yes (vs. no)	−1.54	0.2	0.76	0.05–0.95	**0.042**
Herd size (number of cows ≥ 2 years)	1.83	6.2	0.88	1.11–35.05	**0.037**

(B) Multivariable logistic regression model regarding risk factors for within-herd prevalence				
Variable	Coefficient	SE[51]	95%CI[52]	*p* value
Intercept	0.96	0.24	0.49–1.43	<0.001
Milk feeding Powdered milk (vs. fresh milk)	**−0.68**	**0.24**	**−1.15–0.21**	**0.005**
Heifers sharing alpine pasture with other animal categories of their farm Yes (vs. no)	−0.24	0.17	−0.57–0.09	0.158
Occurrence of diseases in lactating cows at the time of farm visit Yes (vs. no)	**−0.33**	**0.14**	**−0.60–0.05**	**0.022**

[50]OR: Odd Ratio.
[51]SE: Standard Error.
[52]CI: Confidence Interval.
See also Tables 1–3 for definitions of the variables.

knowledge, the only animal that later exhibited signs of JD. There had already been a case of PTB in that herd in 2010 (confirmed by an unidentified laboratory analysis), but no other case had occurred since then until 2022. This underlines the limitations of serum ELISA at a single occasion even if all animals in the herd are tested. The use of more than one test, repeated over time to establish the disease status both of animals and herds, is recommended due to the well known diagnostic limitations for PTB (89). In the present study, every animal could be tested only once with an ELISA test due to practical constraints.

A secondary aim of the study was to identify the subpopulation at highest risk within seropositive herds, i.e., the one that should be preferably tested to determine the PTB status of a herd in the frame of a targeted sampling strategy. This objective could not be achieved given the low proportion of animals with a positive test result in any herd, as only one animal was seropositive in most of them (65.6%). Specific analyses to explore possible associations between individual serostatus and age or fertility (calving interval) of the tested animals did not reveal significant associations (data not shown). Thus, the results of this study suggest that the whole herd should be tested to establish its PTB status. A recommendation as to whether heifers between the age of 1 and 2 years should be tested to determine the PTB status of a herd cannot be made based on the present results, as none of the heifers that tested positive was in a herd that was eventually classified as seropositive. Nevertheless, positive FC results for MAP were found in a surprisingly high proportion of fecal samples from heifers in a previous study in Switzerland, suggesting that younger animals may play a role in the spread of MAP in infected herds and thus should be taken into account when trying to establish a herd status regarding PTB (66). Repeated testing would likely be the key to improve result accuracy (90). Testing of bulk tank milk using commercial ELISA methods is inexpensive and has been investigated for herd screening for JD, however, the results appear to be influenced by herd size and within-herd prevalence (91). Milk ELISA has been proven not to be sensitive enough to detect low prevalence herds (92, 93), thus it does not appear to be an adequate option to identify PTB infected Swiss dairy herds.

The strongest association between a risk factor and seropositive status of the herd was observed for "contact of lactating cows with cows from other herds during grazing" in the multivariable analysis. The lactating cows of three herds (one seropositive and two seronegative) shared grazing pastures around the farm (not alpine pastures) with cows from other herds. In two herds (herd size of 34 and 16 cows, respectively), the cows shared the pasture with cows from two other herds, in the third herd (44 cows) the cows shared the pasture with cows from nine other herds (data not shown). This practice appeared to be a potential risk factor for the herd of testing positive, however, this result must be interpreted with caution given the low number of farms with this characteristic. More data would be needed to further explore this potential risk factor. Nevertheless,

TABLE 8 Farmers' knowledge about paratuberculosis (PTB) in 163 Swiss dairy herds (9 with seropositive and 154 with seronegative status), as assessed by interview.

	Total (%)	Seropositive farms (%)	Seronegative farms (%)
Farmers' awareness of the existence of PTB			
Yes	40 (24.5)	6 (66.7)	34 (22.1)
No	123 (75.5)	3 (33.3)	120 (77.9)
Clinical signs of PTB listed by the farmers aware of the existence of PTB (multiple answers possible)[56]	n answers (%)		
Diarrhea	39 (97.5)	5 (83.3)	34 (100)
Emaciation	30 (75.0)	5 (83.3)	25 (73.5)
Reduction in milk yield	10 (25.0)	2 (33.3)	8 (23.5)
Reduction in fertility	2 (5.0)	0 (0)	2 (5.9)
Other clinical signs mentioned[57]	2 (5.0)	1 (16.6)	1 (2.9)
Transmission pathways of PTB listed by the farmers aware of the existence of PTB (multiple answers possible)[58]			
Direct fecal-oral transmission	16 (40.0)	3 (50.0)	13 (38.2)
Intrauterine infection	13 (32.5)	3 (50.0)	10 (29.4)
Transmission via MAP excretion into the milk of infected cows	7 (17.5)	1 (16.6)	6 (17.6)
Transmission via MAP excretion into the colostrum of infected cows	2 (5.0)	0 (0)	2 (5.9)
Pasture contamination by wild ruminants' feces	1 (2.5)	0 (0)	1 (2.9)
Others transmission ways cited[59]	4 (10.0)	0 (0)	4 (11.8)
No transmission pathways known	18 (45.0)	3 (50.0)	15 (44.1)

[56]Clinical signs of PTB that were cited by the farmers when asked about the disease (open question).
[57]Other clinical signs cited by farmers as possibly associated with PTB (that are actually not directly related to the disease) included abortion and poor coat quality.
[58]Transmission pathways of PTB that were cited by the farmers when asked about the disease (open question).
[59]Other transmission pathways cited by the farmers (that are actually not directly related to PTB) included transmission via air, blood, placenta, and mucous membranes.

keeping manure from cattle from other farms away from the herd by avoiding community or shared pasture has been recommended to decrease the risk of MAP introduction in dairy operations (94). Common pasturing has rather been suggested as a risk factor for young animals (calves and heifers) sharing pastures with adult cattle (95), which is in line with young animals being most susceptible to infection with MAP (6). In the present study, however, the variable "heifers sharing alpine pastures with animals from other farms" (mostly young animals, in 67.5% of the cases, but also with adult animals in 15.3%) was found to be associated with decreased odds of the herd testing seropositive, which was unexpected, especially as MAP shedding in heifers has been reported in a previous study in Switzerland (66). Common alpine pasturing of young animals is widespread in Switzerland (96) and the importance of alpine communal pasturing for the spread of other infectious diseases such as Bovine Virus Diarrhea (BVD) is well documented (97). In our study, heifers sharing alpine pastures with animals from other herds, was reported in 5 of 9 seropositive herds (55.6%, only with young animals) and 130 of 154 seronegative herds (84.4%, of which 68.2% with contact to young animals only and 16.2% to adult cattle), thus potential exposure to MAP may not have been the same in both groups. Although it may be postulated that the significant association may be related to low contact intensity due to extensive pasturing on alpine pastures or to the fact that PTB is not as highly contagious as BVD, these results must, again, be interpreted with caution given the low number of positive farms. Indeed, the odds ratios of all variables remaining in the final multivariable model exhibit wide confidence intervals (Table 7), indicating substantial uncertainty. Consequently, the interpretability of these risk factors is limited. This uncertainty primarily stems from the small number of seropositive herds and risk factors with limited discriminative power due to the presence of only a few observations within specific groups.

The identification of significant risk factors associated with high within-herd prevalence was rendered difficult by the low sensitivity of the ELISA test and the low within-herd prevalence, therefore the results of the analyses conducted to identify such factors must be interpreted with even greater caution than associations with the serostatus of the herds. It is, indeed, difficult to propose biologically rational explanations for the fact that the occurrence of diseases (mostly claw and udder problems were mentioned by the farmers) at the herd level in lactating cows would contribute to decreasing the risk of MAP spread in a positive farm. It must be pointed out that, e.g., relevant diseases in cows at the herd level were not observed in any of the 9 seropositive herds. Likewise, the calves were fed milk powder only (no fresh milk) in 22.2% of the seropositive herds (2/9) but in only 7.1% of the seronegative herds (11/154); this random repartition in our study population might explain, at least in part, why the feeding of milk powder, a common recommendation for PTB positive herds, appeared to be related to a low risk of MAP spreading.

In general, farmers' knowledge about PTB was low, with more than 75.5% of the participants unaware of the very existence of the disease, of which 2.4% turned out having a seropositive herd. This suggest that signs of clinical disease would not be recognized as suspect of PTB by these farmers. After fulfilling the questionnaire,

17.2% of the participants could not exclude that they had had cases of PTB in their herd in the past, while most of them (74.8%) still stated that they had never had a case, although 2.4% of their herds were eventually classified as seropositive. Thirteen of the 163 farmers indicated that they had had at least one case (between one and five diseased animals per farm) in the 10 years prior to the study. While almost all 40 farmers who were aware of the existence of PTB were able to mention the cardinal symptoms of diarrhea (97.5%) and emaciation (75%), only 25% mentioned the reduction in milk yield and 5% also a reduction in fertility. The infection pathways were poorly understood by most, only 35% of the farmers aware of the disease were able to mention at least one infection pathway. The two mostly mentioned pathways were via feces (40%) and intrauterine infection (32.5%). Transmission through milk was rarely known (17.5%) and only 5% of the farmers were aware of possible transmission via colostrum. These results suggest that, despite previous studies in Switzerland (66, 98), PTB is still poorly known by Swiss farmers and further efforts to raise awareness are necessary. It is especially important to raise awareness about transmission through milk, due to the previously mentioned zoonotic potential of MAP, especially in a country with an important tradition in dairy production.

5 Conclusion

The results of the present study revealed a very low between-herd and within-herd prevalence of MAP seropositivity in Swiss dairy farms, even in large herds. This questions the true epidemiologic relevance of PTB in Switzerland. The low within-herd prevalence and the small size of Swiss dairy herds prevented the identification of a target population to be tested preferably to determine the PTB status of a dairy herd. In combination with the low performance, especially the low sensitivity, of serologic tests for the diagnosis of PTB, these results suggest that alternative methods (e.g., repeated PCR of environmental samples) may be more adequate and should be evaluated for Swiss dairy herds. Difficulties in recruiting farmers willing to participate in the study and their low level of awareness of PTB reveal knowledge gaps and poor understanding about infectious diseases, their transmission and the importance of biosecurity measures. Better information and education of Swiss dairy farmers about PTB and biosecurity in general should be a priority in the near future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by Amt für Veterinärwesen, 3000 Bern, Switzerland. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MO: Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. IL: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Conceptualization, Funding acquisition, Resources, Software, Supervision, Validation. JW: Data curation, Formal analysis, Methodology, Software, Supervision, Writing – original draft, Writing – review & editing. SS: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. MS: Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. RSc: Investigation, Writing – original draft, Writing – review & editing. RSt: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. MM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This project was funded by the Swiss Food Safety and Veterinary Office (grant 1.21.05). Open access funding was provided by the University of Bern.

Acknowledgments

We thank Alix Verdillon and Nicole von Arx for assistance with farm visits and sampling, and Sandra Schoch-Müller for technical help in the laboratory.

Conflict of interest

IL and JW were employed by SAFOSO AG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1409694/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 31 July 2024
DOI 10.3389/fvets.2024.1395928

OPEN ACCESS

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RECEIVED 04 March 2024
ACCEPTED 15 July 2024
PUBLISHED 31 July 2024

CITATION
Stefanova EP, Sierra E, Fernández A, Quesada-Canales O, Paz-Sánchez Y, Colom-Rivero A, Espinosa de los Monteros A, Herráez P, Domínguez L, Bezos J, Pérez-Sancho M, Moreno I, Risalde MA and Andrada M (2024) Detection of caprine paratuberculosis (Johne's disease) in pre- and post-vaccinated herds: morphological diagnosis, lesion grading, and bacterial identification. *Front. Vet. Sci.* 11:1395928. doi: 10.3389/fvets.2024.1395928

Detection of caprine paratuberculosis (Johne's disease) in pre- and post-vaccinated herds: morphological diagnosis, lesion grading, and bacterial identification

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Samples from the mesenteric lymph nodes (MS LNs) and ileocecal valves (ICV) of 105 goats, comprising 61 non-vaccinated and 44 vaccinated against *Mycobacterium avium* subspecies *paratuberculosis* (MAP), were collected at slaughter from a farm with a confirmed history of paratuberculosis (PTB). These goats had subclinical infections. PTB-compatible lesions in the MS LNs, ICV lamina propria (LP), and Peyer's patches (PPs) were graded separately. Furthermore, the load of acid-fast bacilli was quantified using Ziehl-Neelsen staining (ZN), MAP antigens by immunohistochemistry (IHC), and MAP DNA by PCR targeting the IS900 sequence. Gross PTB-compatible lesions were found in 39% of the goats, with 31.72% vaccinated (V) and 68.29% non-vaccinated (nV). Histopathological lesions induced MAP were observed in 58% of the animals, with 36.07% vaccinated and 63.93% non-vaccinated. The inclusion of histopathology as a diagnostic tool led to a 28% increase in diagnosed cases in MS LNs and 86.05% in ICV. Grade IV granulomas with central mineralization and necrosis were the most common lesions in MS LNs. In the ICV, mild granulomatous enteritis with multifocal foci of epithelioid macrophages was predominant, occurring more frequently in the PPs than in the LP. Furthermore, statistical differences in the presence of histopathological lesions between vaccinated and non-vaccinated goats were noted in MS LNs, ICV LPs, and ICV PPs. Non-vaccinated animals showed higher positivity rates in ZN, IHC, and PCR tests, underscoring the benefits of anti-MAP vaccination in reducing PTB lesions

and bacterial load in target organs. Our findings emphasize the necessity of integrating gross and histopathological assessments with various laboratory techniques for accurate morphological and etiological diagnosis of PTB in both vaccinated and non-vaccinated goats with subclinical disease. However, further studies are required to refine sampling protocols for subclinical PTB in goats to enhance the consistency of diagnostic tools.

KEYWORDS

Mycobacterium avium subspecies *paratuberculosis*, histopathology, Ziehl-Neelsen, immunohistochemistry, molecular diagnosis, vaccination, goat

1 Introduction

Paratuberculosis (PTB), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a progressive emaciating disease that causes significant economic losses in both cattle and small ruminant industries worldwide (1–5). Although animals usually get infected at an early age due to the high degree of environmental contamination, the subclinical PTB phase is long, and no clinical signs are observed (1–3, 5, 6). Furthermore, once an animal enters the clinical PTB stage, the signs exhibited are non-specific, including diarrhea and severe weight loss (1–3, 5, 6).

Currently, the European Union counts 11,262 thousand goat heads, with the Spanish population being the second largest, with 2,463 thousand heads registered by the end of 2022 (EUROSTAT). The Canary Islands archipelago has the fourth largest goat population in Spain (MAPA 2022), with 232,060 heads and 1,256 farms (ISTAC 2023) of mainly certified autochthonous endangered breeds (Orden APM/26/2018). The overall annual mortality on the canary goat farms in 2017 was approximately 20%, of which 38% was due to PTB (Jiménez, 2017, unpublished data). Although data about the exact prevalence of goat PTB in the continental part of Spain is limited, it is considered a widespread disease that causes considerable economic losses throughout the country (2, 7).

PTB control strategies on goat farms include testing, culling, and vaccination (8). It is demonstrated that vaccination against MAP may considerably reduce the disease-originated economic losses in industrial farms, as well as the severity of the clinical signs and pathological lesions in target organs, although its use has raised controversy since cross-reaction with tuberculosis (TB) on-field diagnostic tests has been described (6, 9–11). Various whole cells were killed, and live attenuated and inactivated vaccines have been developed to prevent PTB in ruminants (6–8, 11–17).

According to the local legislation in the Canary Islands (Decreto 51/2018 del 23 de abril), caprine vaccination is only permitted after "TB-free" status confirmation and PTB diagnostic confirmation since the region was declared "officially free" of bovine TB in 2017. The official testing guidelines establish the comparative intradermal tuberculin (CIT) test as a mandatory exam.

TB and PTB are important livestock concerns worldwide, and goats are especially sensitive to both diseases, with co-infection being reported (18). Thus, proper herd diagnosis is a crucial part of control and prevention strategies (19). Different laboratory tests are used at the herd level for diagnostic confirmation. Indicative clinical signs, evaluation of the host immune response, and necropsy performance are among the commonly used tools. Microbiological culture is the gold standard for PTB detection, but its accuracy is limited in the early PTB stages (19). Molecular biology techniques such as polymerase chain reaction (PCR) targeting the insertion sequence 900 (IS900) have proven to be one of the most sensitive methods for the detection of MAP DNA in different samples, including affected tissues, feces, milk, and buffy coat, although some authors state that its sensitivity decreases when the bacterial load is low (8, 12, 20–22).

Pathological examination is an important tool for adequate confirmation of a postmortem herd diagnosis. Correct evaluation of gross and histopathological findings in tissue samples from affected goats is of utmost importance for a correct diagnosis (12, 23). Routine necropsies are commonly performed to obtain samples for laboratory confirmation and on-field diagnosis via postmortem examination. Gross lesions originating from MAP presence are usually confined to the intestinal mucosa and the draining lymph nodes (12, 24, 25). The intestinal mucosa can be affected by segmental or diffuse lesions distributed from the duodenum to the rectum, although the sites usually affected are the lower ileum and the upper large intestine. The ileocecal valve (ICV) is important for the correct diagnosis, although the detection of gross lesions in this region is variable. The mesenteric (MS) and ileocecal (IC) lymph nodes (LNs) are diffusely enlarged, edematous, and pale. Lymphangitis, which in mild cases can be the only gross lesion present, can be detected with lymphatic vessels being traceable as thickened cords through the MS LNs, as well as intestinal serosa and mesentery (24).

On the other hand, the main MAP-induced histopathological lesions in goats involve transmural granulomatous enteritis, mesenteric and ileocecal granulomatous lymphadenitis, and lymphangitis (12, 24, 25). Intestinal villi can appear moderately to markedly shortened with the infiltration of epithelioid macrophages and a variable number of Langhans-type multinucleated giant cells. The granulomatous infiltrate can have focal or diffuse distribution along the lamina propria, submucosa, muscular layer, or serosa of the intestine. Well-formed granulomas can form and project into the lumen, and in some cases, they undergo central necrosis. The LN lesions can also vary, ranging from foci of epithelioid macrophages up to the final stage of encapsulated granulomas with central necrosis (12, 24, 25).

Since gross lesions are not always present in caprine PTB, correct histopathological assessment is indispensable for adequate postmortem diagnosis of clinical and subclinical cases (7, 12, 23, 26, 27). Thus, various grading systems have been proposed based

on the severity and extension of the lesions, the predominant cell type, and/or the bacterial load (12, 13, 28–31). In the case of bovine and ovine PTB, different gradings have been described in both experimental studies and natural infection (13, 29–31). Nevertheless, in the case of natural caprine PTB, the widely used grading proposed by Corpa et al. is based on both the histopathological findings and Ziehl-Neelsen (ZN) quantification (12). To our knowledge, no grading system has been applied in natural cases of goat PTB to evaluate the histopathological findings and the bacterial load separately.

The aim of the present study is to evaluate and grade the PTB-compatible lesions in subclinical goat cases detected at slaughter. Further emphasis is placed on the effects of the anti-MAP vaccination on the development of lesions and the use of grading to optimize early diagnosis. Furthermore, we assess the diagnostic performance of histochemical, immunohistochemical, and molecular techniques for PTB detection in both vaccinated (V) and non-vaccinated (nV) subclinical caprine cases.

2 Materials and methods

2.1 Herd history

As part of the authorization process for vaccination against PTB, this study was conducted on an intensive dairy goat farm with a census of 3,090 heads located in the Canary Islands, Spain. The local legislation (Decreto 51/2018 del 23 de abril) requires confirmation of PTB presence in the herd as well as certification of TB-free status to grant a vaccination permit. The details are explained in the following sections.

2.1.1 PTB confirmation

The presence of PTB was confirmed at the herd level, as indicated by clinical signs such as weight loss, poor body condition, decreased daily milk production, and diarrhea. More than 50% of the animals tested positive for avian purified protein derivative (aPPD) via the CIT tests. Additionally, serological assays performed on blood serum using the PARACHEK® 2 Kit (Thermo Fisher Scientific, Massachusetts, USA) detected anti-MAP antibodies in over 20% of the animals. Finally, using PCR, MAP DNA was isolated in 35 of the 40 environmental samples tested, contributing to the verification of a TB-free status.

2.1.2 TB-free status confirmation

The absence of TB in the herd was confirmed as requested by the local authorities. A P22 antigenic complex ELISA (Sabiotec, Ciudad Real, Spain) (32) was carried out to detect antibodies against the *Mycobacterium tuberculosis* complex in blood serum. All the results were negative. Moreover, a total of 40 environmental samples were collected using dry sponges (3 M™ Dry-Sponge; 3 M-España, Madrid). Subsequently, DNA was extracted, and PCR was performed on the environmental samples. None of those tested positive for the *Mycobacterium tuberculosis* complex. Finally, an on-field compared CIT test for detection of the *Mycobacterium tuberculosis* complex was performed as requested by the local legislation. All animals with positive or inconclusive results were sent to slaughter. The absence of TB in those animals was confirmed by histopathology and bacterial culture performed by the laboratory of VISAVET, Health Surveillance Centre, Madrid, Spain.

2.2 Animals

A total of 105 goats (61 non-vaccinated and 44 vaccinated) were sampled, including 86 animals with positive/inconclusive results on the CIT test and 19 goats with negative results. All of them were euthanized as part of the TB-free status confirmation protocol. Sampling was conducted in two sessions: the first before vaccination, as mandated by local legislation, and a second session 9 months after vaccination implementation. None of the studied animals exhibited PTB-compatible clinical signs, such as severe emaciation, protrusion of lumbar vertebrae, easily palpable transverse processes, muscle mass loss, or reduction in visceral fat deposits. Thus, all cases were classified as "subclinical".

2.3 CIT test

The CIT test was conducted on the farm as per the methodology outlined in previous studies (33), adhering to the Spanish national legislation, R.D. 2611/1996, the European Regulation, EU 2016/429, and the Commission Delegated Regulation EU 2020/688, Orden del 29 de Abril de 2002 de la Consejería de Agricultura y Ganadería de Castilla y León, and Orden AYG/894/2010. Bovine purified protein derivative (bPPD) (0.1 mL; CZ Vaccines S.A., O Porriño, Pontevedra, Spain) was administered on the left side of the neck and avian PPD (0.1 mL; CZ Vaccines S.A., O Porriño, Pontevedra, Spain) on the right side. Readings were taken 72 h post-injection. A positive TB result was indicated by an increase in skinfold thickness at the bPPD injection site on the left side of the neck exceeding 4 mm more than the reaction at the avian PPD site. An 'inconclusive' result for TB was noted if the increase in skin fold thickness at the bPPD site was 8 mm or more or was equal to or less than the reaction at the avian site. Any other reactions were classified as negative.

2.4 Anti-MAP vaccine

In the present study, an anti-MAP Gudair® commercial heat-inactivated vaccine containing 2.5 mg/mL of MAP strain 316 F with mineral oil adjuvant (CZ Vaccines S.A., O Porriño, Pontevedra, Spain) for use in sheep and goats was applied. It was administered once subcutaneously following the manufacturer's instructions and the guidelines of the Spanish Agency for Medicines and Medical Devices (AEMPS), which indicate that in heavily affected herds, all animals, including adult ones, should be vaccinated.

2.5 Gross examination

An experienced pathologist conducted a macroscopic evaluation of all tissues sampled at the slaughterhouse. Gross lesions were recorded and described in terms of location, color, size, shape, consistency, and number or percent of involvement of the affected organ (34). Subsequently, a presumed morphological diagnosis was established.

2.6 Sample collection and processing for histological examination

Samples were collected immediately after the routine slaughter in an abattoir using standard authorized methods detailed in the Spanish national legislation (R.D. 37/2014). LNs and intestines were sampled for histology according to the recommendations of the national bovine TB eradication program protocol 2021 (35) as follows: MS, IC, retropharyngeal (RPh), prescapular (PE), and mediastinal (MD) LNs and ICV. Fresh tissue samples for molecular biology were frozen at −20°C.

For histopathologic examination, tissue samples were fixed in 10% buffered formalin, embedded in paraffin, processed routinely, sectioned at 4 μm, and stained with hematoxylin/eosin (HE).

2.7 Histological evaluation of MAP-induced lesions

PTB-compatible histological lesions were graded separately in the MS LNs and the ICV, applying two grading systems. PTB-compatible granulomatous lymphadenitis, ranging from focal to multifocal accumulation of epithelioid macrophages with or without multinucleated giant cells up to encapsulated, well-formed granuloma with central necrosis and mineralization, was classified into four stages. The guidelines followed were the ones described by Wangoo et al. for grading TB LN granulomas: 0 (no lesions), I (initial), II (solid), III (minimal necrosis), and IV (necrosis and mineralization) (36). The ICV affections were graded in terms of severity (mild, moderate, and marked) and distribution (focal, multifocal, and diffuse) using the grading system proposed by Krüger et al., evaluating the lamina propria (LP) and Peyer's patches (PPs) (23) separately. Severity was considered mild when only small circumscribed granulomatous infiltrates were present with no change of tissue architecture; moderate when granulomatous infiltrates with altered tissue architecture were present; and marked when massive granulomatous infiltrates with partially or completely disrupted tissue architecture were observed. The distribution of the lesions was evaluated as focal when up to three distinct granulomatous infiltrates were observed per section, multifocal when more than three distinct granulomatous infiltrates per section were observed, and diffuse when granulomatous infiltrates were present throughout the whole section.

2.8 ZN stain and immunohistochemistry (IHC) for MAP confirmation

Selected tissue samples from MS LNs and ICV were sectioned at 4 μm and stained with ZN to detect acid-fast bacilli (AFB) (37).

For immunohistochemistry, samples from the MS LNs and the ICV were sectioned at 3 μm. No epitope retrieval was needed. Inactivation of the endogenous peroxidase was carried out using a solution of 3% hydrogen peroxide in methanol for 30 min in a humidified chamber. Subsequently, immunohistochemical labeling was conducted with a polyclonal anti-MAP in-house antibody kindly provided by Dr. V. Pérez, University of León, León, Spain, diluted 1:2,000 in an antibody diluent (K8006; Dako, Glostrup, Denmark). Sections of ICV samples from PCR-positive MAP-infected goats with PTB histopathological lesions were used as a positive control. The polymer-based detection system that was used (EnVision®System Labeled Polymer-HRP; Dako, Glostrup, Denmark) was applied following the manufacturer's guidelines. A commercial solution of 3,3′diaminobenzidine (DAB) (K3468; Dako, Glostrup, Denmark) was used for immunolabelling, and finally, the sections were counterstained with Harris' hematoxylin and mounted in a hydrophobic medium.

The grading score applied to evaluate the number of mycobacteria per section by both IHC and ZN was the one proposed by Krüger et al. (23). Samples were graded as negative when <2 labeled bacteria were observed per section; as "single" when mycobacteria were present in <20% epithelioid cells and/or multinucleated giant cells (MGCs) and single/few bacteria per cell or foci of granular labeling were observed, predominantly <21 μm in diameter; as "few" when mycobacteria was detected in 20% to 50% epithelioid cells and/or MGCs and on average, 1–10 bacteria were present per cell or foci of granular labeling predominantly >21 μmin diameter; and as "many" when mycobacteria was seen in >50% to 75% epithelioid cells and/or MGCs with an average of >10 bacteria per cell, with up to 50% of cells containing countless bacteria (23).

2.9 MAP DNA identification

Frozen tissue samples from the MS LNs and ICV were included in a pool sample for DNA extraction. DNA was isolated using the quick-DNA/RNA Magbead extraction kit (Zymo, Irvine, CA, US) following the manufacturer's protocol, which was carried out using the automated extractor TECAN Freedom EVO 200 (Tecan Australia Pty Ltd.).

Subsequently, real-time PCR targeting the IS900 sequence was performed following the protocol previously described by Espinosa et al. (8). Briefly, a mixture of 0.5 μL of 250 nM of forward (MP10-1, [5′-ATGCGCCACGACTTGCAGCCT-3′]) and reverse (MP11-1, [5′-GGCACGGCTCTTGTTGTAGTCG-3′]) primers, 10 μL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, CA, USA), 2 μL of DNA template, and nuclease-free water caused the final volume to be 20 μL. The amplification involved one cycle of 95°C for 8.5 min, 40 cycles of 95°C for 15 s, 68°C for 30 s, and melt curve analysis from 72°C to 95°C using a MiniOpticon™ Real-Time PCR System (Bio-Rad Laboratories, Irvine, CA, USA).

Samples were considered positive when the dissociation peak (Tm) was 89.1 ± 1.5°C, and the threshold cycles (Ct) were ≤ 37. The real-time PCR was performed in triplicate to exclude the negative pool samples.

The PCR products from positive pool samples were purified using a commercial kit (Real Clean Spin Kit 50 Test-REAL) following the manufacturer's protocol. Subsequently, those were sequenced using Sanger DNA sequencing (Secugen S.L., Madrid, Spain). The BLAST database (www.ncbi.nlm.nih.gov/blast/Blast.cgi/) was used to confirm the amplicon identities.

2.10 Statistical analysis

An observational cross-sectional study was carried out. The prevalence of macroscopic and microscopic PTB-compatible lesions and their histopathological grading were calculated separately in the MS LNs, the ICV LP, and the ICV PPs.

Statistical analysis was conducted using IBM SPSS Statistics 27 (IBM Corp., released 2020). IBM SPSS Statistics for Windows, Version 27.0. (Armonk, NY, USA: IBM Corp.). Categorical variables were presented as percentages and either relative or absolute frequencies. The ages of the animals were grouped into the following groups: 0–12 months, 12–24 months, 24–36 months, 36–48 months, and >48 months. The association between two categorical variables was analyzed using the Chi-square test, while Kendall's Tau-b test was employed for two ordinal scale variables. Numerical variables were summarized by their mean, standard deviation (SD), median, and interquartile range (IQR). The Shapiro–Wilk test was used to assess the normality of the sample. For non-normal distributions, the Mann–Whitney U test was applied to compare two independent samples. The agreement between qualitative results from two measurement procedures was evaluated using the kappa coefficient (κ). Results were deemed statistically significant if the $p < 0.05$.

3 Results

The ages of the goats ranged from 6 to 101 months, with a median age of 14 months, a standard deviation (SD) of 17.60 months, and an interquartile range (IQR) of 7 months. The distribution of goats across age groups was as follows: 31 out of 105 goats were in the 0–12 month group, 53 in the 12–24 month group, 7 in the 24–36 month group, 3 in the 36–48 month group, and 11 in the over 48 month group. Overall, 80% (84 out of 105) of the goats, including 78.70% (48 out of 61) nV and 81.82% (36 out of 44) V animals, were aged between 0 and 24 months.

3.1 CIT test

A total of 47.62% of the goats tested positive for the *Mycobacterium tuberculosis* complex using the CIT test, with 34.29% categorized as "inconclusive" and 18.10% categorized as negative. No statistical difference was demonstrated between the ages of the animals and the CIT test results.

The inconclusive group comprised 34.29% of the sample, with a higher proportion of nV animals (83.33%) compared to vaccinated (V) animals (16.67%), a difference that was statistically significant ($p = 0.001$). In contrast, no significant differences were found between the CIT-negative and CIT-positive animals ($p = 0.405$). Animals with PTB-compatible lesions were identified across the CIT-positive, inconclusive, and negative groups. The association between the CIT test results and the presence of PTB lesions is further explored in Section 3.4.

3.2 Gross PTB-compatible lesions

PTB-compatible gross lesions affecting the MS LNs and/or the ICV were found in 39.00% of the animals, of which 68.29% were nV and 31.72% were V (Figure 1). No statistical differences were demonstrated in relation to vaccination status. Gross granulomatous lymphadenitis ranging from small focal granulomas (<5 mm) with minimal mineralization (Figure 1A) up to well-formed encapsulated calcified multifocal coalescent granulomas (Figure 1C) was observed in 37.14% of animals. On the other hand, the mucosa of the ICV appeared thickened, corrugated, and folded into transverse rugae in 6.67% (Figure 1D) of the goats, in contrast with 93.33% of the animals in which the ICV had no apparent gross lesions (Figure 1B).

3.3 Histopathological PTB lesions

The main histopathological findings compatible with PTB are summarized in Table 1, Figures 2, 3. Granulomatous lesions affecting the MS LNs and/or the ICV were detected in 58.10% of the animals, 36.07% were V, and 63.93% were nV ($p = 0.013$). Both MS LNs and the ICV were affected in 52.46% of the animals, with 81.25% nV and 18.75% V (Figure 3A).

The MS LNs presented granulomatous lymphadenitis with microgranuloma and granuloma formation in 47.62% of the goats, and the ICV had granulomatous enteritis with epithelioid macrophages and occasional multinucleated giant cells in LP and/or PPs in 40.95%. Thus, histopathological examination contributed to diagnosing more affected animals in which no gross lesions were described. Specifically, the increase in diagnosed cases was 28% in MS LNs and 86.05% in ICV. Regarding the vaccination status, the V animals appeared less affected histologically in MS LNs ($p = 0.018$), ICV LP ($p = 0.027$), and ICV PPs ($p = 0.028$). In relation to the grading of the PTB-compatible lesions affecting the MS LNs, grade IV encapsulated granulomas with mineralization (Figure 2A) were the most frequent finding (60%), mainly present in the nV group (70%), although the difference was not statistically significant ($p = 0.068$).

In the case of the ICV, the lesions in LP and the PPs were graded as multifocal in 86.96% and 79.49% and mild in 95.65% and 94.87%, respectively (Figures 2B, C). A greater number of nV goats presented lesions in both LP and PPs, with a statistical difference also being demonstrated in terms of grading (Table 1).

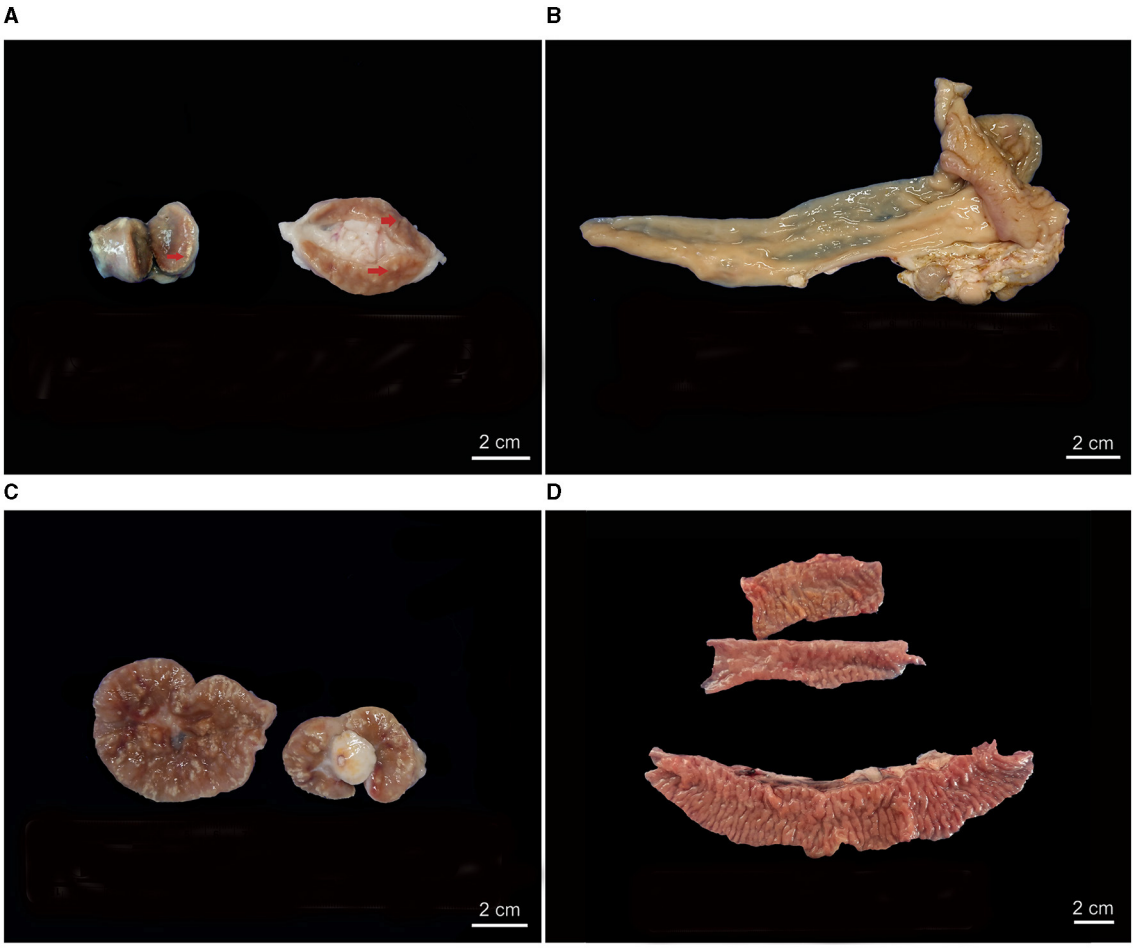

FIGURE 1
Gross PTB-compatible lesions in vaccinated (V) and non-vaccinated (nV) goats. **(A)** Lymphadenomegaly of mesenteric lymph nodes (MS LNs) of a V animal with multifocal white calcified granulomas on the cut surface (arrow). **(B)** Ileocecal valve (ICV) of a V animal with no gross lesions. **(C)** MS LNs and mesenteric fat of an nV animal exhibiting numerous multifocal to coalescing white calcified granulomas on the cut surface. **(D)** ICV of an nV animal showing diffusely thickened mucosa folded into transverse rugae, characteristic of PTB.

3.4 Relationship between CIT-test results and PTB-compatible lesions

The main differences were observed in the groups of goats with "inconclusive" CIT test results. More nV goats from this group (66.67%) presented PTB-compatible gross lesions affecting the MS LNs and/or the ICV ($p = 0.005$). Regarding the histopathology results of the same nV animals, 83.33% presented PTB-compatible granulomatous lymphadenitis of the MS LNs and/or granulomatous enteritis affecting the ICV ($p = 0.008$). Thus, 15.67% more nV animals were detected as possibly affected by PTB by adding the use of histopathology as a diagnostic tool. No such difference was demonstrated in the V group ($p = 0.112$), in which only 16.67% of the animals with inconclusive CIT test results had gross PTB-compatible lesions.

In the case of the animals who tested "positive" to the CIT test (47.62%), 52% were V and 48% nV. Gross PTB-compatible lesions were described in 38.46% of the V and in 29.17% of the nV animals. Histopathological PTB-compatible lesions were found in 61.54% of the V and in 45.83% of the nV. No differences were demonstrated regarding the presence of gross or histopathological lesions between the two groups.

In the CIT-negative animals (18.10%), 63.16% of the goats were V and 36.84% nV. Gross PTB-compatible lesions were observed in 16.67% of the V and in 14.29% of the nV. Furthermore, applying histopathology as a diagnostic tool, 41.67% of the V animals and 42.86% of the nV were categorized as goats with PTB-compatible lesions in MS LNs and/or ICV.

3.5 MAP identification

3.5.1 ZN staining

The main results of the ZN stain are summarized in Table 2. Red acid-fast bacilli (AFB) were identified in the cytoplasm of epithelioid macrophages, multinucleated giant cells, and/or the mineralized centers of well-formed encapsulated granulomas affecting the MS LNs and/or the ICV in 22.86% of the animals, 12.50% of which were V and 87.50% were nV (p=0.001). A total of 34.43% of the nV goats had PTB-compatible lesions in MS LNs

TABLE 1 Summary of histopathological PTB-compatible lesions in 105 naturally infected goats (vaccinated and non-vaccinated).

Lesion site	Lesion grading	Animals with histopathological; PTB-compatible lesions (%)	V	nV	Statistical analysis between groups (*p*-value)*
MS LNs and/or ICV		61 (58.10%)	22 (36.07%)	39 (63.93%)	**0.013**
MS LNs		50 (47.62%)	15 (30%)	35 (70%)	**0.018**
	Grade I	14 (28%)	6 (42.86%)	8 (57.14%)	**0.068**
	Grade II	4 (8%)	-	4 (100%)	
	Grade III	2 (4%)	-	2 (100%)	
	Grade IV	30 (60%)	9 (30%)	21 (70%)	
ICV LP and/or PPs		43 (40.95%)	13 (30.23%)	30 (69.77%)	**0.044**
ICV LP		23 (21.90%)	5 (21.74%)	18 (78.26%)	**0.027**
Distribution	Focal	2 (8.70%)	2 (100%)	-	**0.004; 0.037****
	Multifocal	20 (86.96%)	2 (10%)	18 (90%)	
	Diffuse	1 (4.35%)	1 (100%)	-	
Severity	Mild	22 (95.65%)	4 (18.18%)	18 (81.82%)	**0.023; 0.037*****
	Moderate	1 (4.35%)	1 (100%)	-	
	Severe	-	-	-	
ICV PPs (*n* = 97)		39 (40.21%)	11 (28.21%)	28 (71.79%)	**0.028**
Distribution	Focal	8 (20.51%)	6 (75%)	2 (25%)	**0.001**
	Multifocal	31 (79.49%)	5 (16.13%)	26 (83.87%)	
	Diffuse	-	-	-	
Severity	Mild	37 (94.87%)	11 (29.73%)	26 (70.27%)	**0.034**
	Moderate	2 (5.13%)	-	2 (100%)	
	Severe	-	-	-	

nV, non-vaccinated; V, vaccinated; MS LNs, mesenteric lymph nodes; ICV, ileocecal valve region; LP, lamina propria; PPs, Peyer's patches.
*The results were considered statistically significant if the $p < 0.05$.
**Significant association between the ages and the severity of PTB-compatible histopathological lesions affecting the ICV LP in the nV group.
***Significant association between the ages and the distribution of PTB-compatible histopathological lesions affecting the ICV in the nV group.
The bold text represents statistically significant differences.

and/or ICV and tested positive on ZN, in contrast with only 6.82% of the V (p=0.001). A total of 63.64% of the ZN-positive animals presented AFB in both MS LNs and ICV, with 14.29% of those V and 85.71% nV (Figure 3B). The ZN positivity was compared between the V and nV animals. Fewer V animals were ZN positive in MS LNs (p=0.004) and in the ICV LP and/or PPs (p=0.006). The AFB load was graded as "single" in 80.95% of the ZN-positive MS LNs, of which only 17.65% were from V animals and 82.35% from nV (p=0.013) (Figure 2D). Regarding the bacterial load in the ICV, 6 of the cases had "single" AFB, 1 had "few," and 2 had "many" (Figure 2F). The bacterial load in the PPs was graded as "single" in 84.62%, 90.91% of which were from nV animals. Only two goats had "few" (Figure 2E) or "many" AFB, and both were nV.

3.5.2 Immunohistochemistry (IHC)

The main results of the IHC stain are summarized in Table 2. An immunoreaction seen as brown cytoplasmic granules in epithelioid macrophages and/or multinucleated giant cells in lesions affecting the MS LNs and/or the ICV was detected in 33.33% of the studied animals, being only 14.29% V and 85.71% nV ($p = 0.001$). The presence of histopathological PTB lesions and the IHC positivity were compared between the V and nV groups, and statistical differences were demonstrated in animals with lesions in MS LNs and/or ICV ($p = 0.001$) and in those with lesions affecting the ICV LP ($p = 0.001$). Both MS LNs and ICV were IHC positive in 45.71% of the goats, of which 6.25% were V, and 93.75% were nV (Figure 3C).

The differences between the V and nV animals regarding IHC positivity were further analyzed. Fewer V animals were IHC positive in MS LNs ($p = 0.001$), ICV LP, and/or PPs ($p = 0.001$). The MAP load in MS LNs was graded as "single" in 86.67% of the cases, with only 11.54% from V animals and 88.46% from nV ($p = 0.001$) (Figure 2G). Regarding the ICV LP, only two animals presented MAP loads graded as "many" (Figure 2I). In the PPs, 88.24% of the positive samples presented "single" MAP, 93.33% from nV animals and only 6.67% from V ($p = 0.011$), and "few" mycobacteria were detected in only two samples (Figure 2H).

The agreement between the detection capacity of ZN and IHC was moderate ($\kappa = 0.419$). Regarding the detection capacity in the

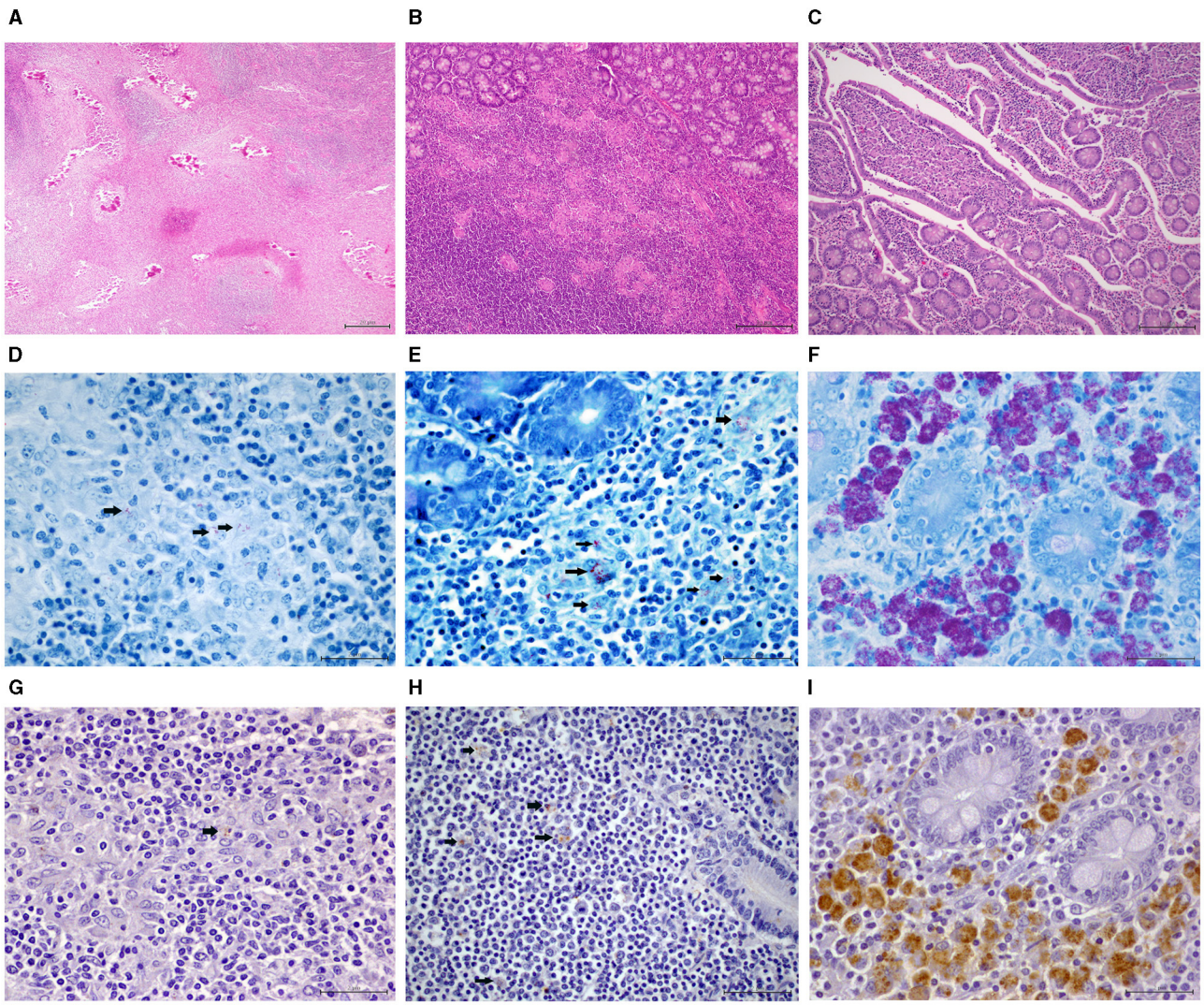

FIGURE 2
Histopathological PTB-compatible findings in vaccinated (V) and non-vaccinated (nV) goats. **(A)** Mesenteric lymph node (MS LN): grade IV lesions with multifocal severe calcified granulomas. HE 4x **(B)** Peyer's patches (PPs) of the ileocecal valve region (ICV): multifocal mild granulomatous infiltrate of epitheliod macrophages. HE 10x **(C)** Lamina propria (LP) of the ICV: multifocal mild enteritis with epithelioid macrophage aggregates in the tips of the intestinal villi. HE 10x **(D)** MS LN: single acid-fast bacilli (AFB) **(arrows)** present in <20% epithelioid cells and single/few bacteria per cell. Ziehl-Neelsen (ZN) staining, 60x **(E)** ICV, PPs: few AFB detected in 20% to 50% epithelioid cells and multinucleated giant cells (MGCs) with 1 to 10 bacteria present per cell (**arrows**). ZN, 60x **(F)** ICV, LP: AFB is present in >50% to 75% of epithelioid cells and MGCs, with an average of >10 bacteria per cell. ZN, 60x **(G)** MS LN: single mycobacteria (**arrow**) observed in <20% of the epithelioid macrophages. Anti-MAP immunohistochemistry (IHC), 60x **(H)** ICV, PPs: few mycobacteria (**arrows**) detected in 20% to 50% epithelioid cells. IHC, 60x **(I)** ICV, LP: many mycobacteria with strong immunolabeling in >50%–75% epithelioid cells, with approximately 50% of cells containing countless bacteria. IHC, 60x.

MS LNs, the agreement between the two techniques was moderate ($\kappa = 0.513$) in contrast with the results from the ICV samples, where the agreement was low ($\kappa = 0.231$).

3.5.3 Real-time PCR

A total of 28.57% of the pool samples from mesenteric lymph nodes (MS LNs) and ileocecal valves (ICV) tested positive for the presence of MAP DNA, with 26.63% from V goats, and 73.33% from nV goats ($p = 0.001$) (Table 2). Regarding the presence of PTB-compatible lesions, an association was found only in the nV group, in which only one animal tested positive for PCR but had no MAP-induced lesions in target organs ($p = 0.001$). In the V group, only eight animals tested positive for PCR, and thus, no statistical analysis was conducted due to the small sample size. Nevertheless, five of those goats presented MAP-induced lesions in target organs, while no lesions were found in three.

3.5.4 Relationship between the diagnostic techniques for MAP identification (ZN, IHC, and real-time PCR)

Overall, 52.38% of the animals tested negative by the three techniques for MAP identification; 58.18% of those were V goats,

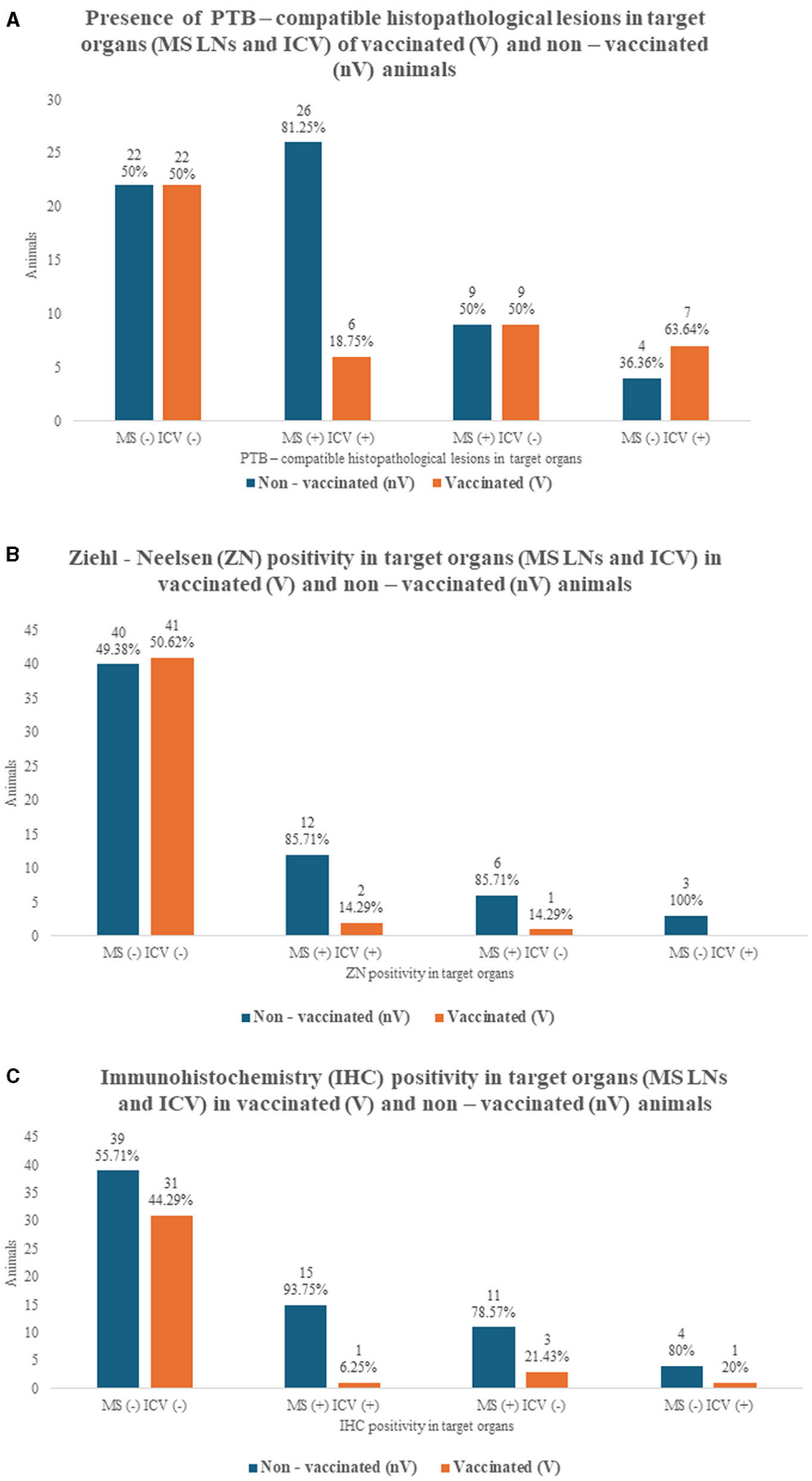

FIGURE 3
Histopathological PTB-compatible lesions, Ziehl-Neelsen (ZN), and immunohistochemistry (IHC) in target organs of 105 naturally infected goats (vaccinated and non-vaccinated). **(A)** PTB-compatible histopathological findings in mesenteric lymph nodes (MS LNs) and/or ileocecal valve region (ICV) of vaccinated (V) and non-vaccinated (nV) goats. **(B)** ZN positivity in MS LNs and/or ICV of V and nV goats. **(C)** IHC positivity in MS LNs and/or ICV of V goats and nV goats.

TABLE 2 Summary of diagnostic test results for the identification of *Mycobacterium avium* subspecies *paratuberculosis* in 105 naturally infected goats (vaccinated and non-vaccinated).

Lesion site	Grading	Positive results (%)	V	nV	Statistical analysis between groups (p-value)*
Ziehl-Neelsen staining					
MS LNs and/or ICV		24 (22.86%)	3 (12.50%)	21 (87.50%)	**0.001**
MS LNs		21 (20.00%)	3 (14.21%)	18 (85.71%)	**0.004**
	Single	17 (80.95%)	3 (17.65%)	14 (82.35%)	**0.013**
	Few	4 (19.05%)	-	4 (100%)	
	Many	-	-	-	
ICV LP and/or PPs		17 (16.19%)	2 (11.76%)	15 (88.24%)	**0.006**
ICV LP		9 (8.57%)	2 (22.22%)	7 (77.78%)	-
	Single	6 (66.67%)	1 (16.67%)	5 (83.33%)	-
	Few	1 (11.11%)	-	1 (100%)	
	Many	2 (22.22%)	1 (50%)	1 (50%)	
ICV PPs (n=98)		13 (13.27%)	1(7.69%)	12 (92.31%)	**0.028**
	Single	11 (84.62%)	1 (9.09%)	10 (90.91%)	0.076
	Few	1 (7.69%)	-	1 (100%)	
	Many	1 (7.69%)	-	1 (100%)	
Immunohistochemistry					
MS LNs and/or ICV		35 (33.33%)	5 (14.29%) **	30 (85.71%)***	**0.001; 0.018**; 0.001*** **
MS LNs		30 (28.57%)	4 (13.33%)	26 (86.67%)	**0.001**
	Single	26 (86.67%)	3 (11.54%)	23 (88.46%)	**0.001**
	Few	4 (13.33%)	1 (25%)	3 (75%)	
	Many	-	-	-	
ICV LP and/or PPs		21 (20%)	2 (9.52%)	19 (90.48%)	**0.001**
ICV LP		15 (14.29%)	2 (13.33%)	13 (86.67%)	**0.015**
	Single	9 (60%)	-	9 (100%)	**0.049**
	Few	4 (26.67%)	1 (25%)	3 (75%)	
	Many	2 (13.33%)	1 (50%)	1 (50%)	
ICV PPs (n=96)		17 (17.71%)	2 (11.76%)	15 (88.24%)	**0.006**
	Single	15 (88.24%)	1 (6.67%)	14 (93.33%)	**0.011**
	Few	2 (11.76%)	1 (50%)	1 (50%)	
	Many	-	-	-	
PCR					
MS LNs and/or ICV		30 (28.57%)	8 (26.67%)	22 (73.33%)	**0.045**

nV, non-vaccinated; V, vaccinated; MS LNs, mesenteric lymph nodes; ICV, ileocecal valve region; LP, lamina propria; PPs, Peyer's patches.
*The results were considered statistically significant if the p-value < 0.05.
**Fewer V animals with PTB-compatible histopathological lesions tested positive for immunohistochemistry.
***More nV animals with PTB-compatible histopathological lesions tested positive for immunohistochemistry.

and 41.82% were nV goats (Table 3). 30.91% of the samples that were identified as negative were from animals that presented histopathological lesions compatible with PTB. On the other hand, 11.43% of the animals tested positive by ZN, IHC, and PCR, 16.67% were V goats, and 83.33% were nV goats. All goats in this group presented MAP-induced lesions in target organs. Thus, the three techniques agree in the identification of both positive and negative animals in 63.81% of the cases, 50.75% from V goats, and 49.25% from nV goats. The agreement between PCR and the ZN was low ($\kappa = 0.355$), and between PCR and IHC was moderate ($\kappa = 0.444$).

The animals identified as positive by at least one of the three techniques were 36.19%, 26.32% were V goats, and 73.68% were nV goats. Only 6 (three V goats and three nV goats) of those animals did not present PTB-compatible histopathological

TABLE 3 Summary of diagnostic results for MAP identification and presence of histopathological PTB-compatible lesions in 105 naturally infected goats (vaccinated and non-vaccinated).

MAP identification techniques	Histopathological PTB-compatible lesions	V	nV	Total; $N = 105$
Agreement between three techniques				
PCR (-) ZN (-)IHC (-)		32 (58.18%)	23 (41.82%)	55 (52.38%)
	Presence	13 (76.47%)	4 (23.53%)	17 (30.91%)
	Absence	19 (50%)	19 (50%)	38 (69.09%)
PCR (+) ZN (+) IHC (+)		2 (16.67%)	10 (83.33%)	12 (11.43%)
	Presence	2 (16.67%)	10 (83.33%)	12 (100%)
	Absence	-	-	-
Subtotal		34 (50.75%)	33 (49.25%)	67 (63.81%)
	Presence	15 (51.72%)	14 (48.28%)	29 (43.28%)
	Absence	19 (50%)	19 (50%)	38 (56.72%)
Agreement between two techniques				
PCR (+) ZN (+) IHC (-)		-	2 (100%)	2 (1.90%)
	Presence	-	2 (100%)	2 (100%)
	Absence	-	-	-
PCR (+) ZN (-) IHC (+)		-	8 (100%)	8 (7.62%)
	Presence	-	7 (100%)	7 (87.50%)
	Absence	-	1 (100%)	1 (12.50%)
PCR (+) ZN (-) IHC (-)		6 (75%)	2 (25%)	8 (7.62%)
	Presence	3 (60%)	2 (40%)	5 (62.50%)
	Absence	3 (100%)	-	3 (37.50%)
PCR (-) ZN (+) IHC (+)		-	5 (100%)	5 (4.76%)
	Presence	-	5 (100%)	5 (100%)
	Absence	-	-	-
PCR (-) ZN (-) IHC (+)		3 (30%)	7 (70%)	10 (9.52%)
	Presence	3 (37.50%)	5 (62.50%)	8 (80%)
	Absence	-	2 (100%)	2 (20%)
PCR (-) ZN (+) IHC (-)		1 (20%)	4 (80%)	5 (4.76%)
	Presence	1 (20%)	4 (80%)	5 (100%)
	Absence	-	-	-
Subtotal		10 (26.32%)	28 (73.68%)	38 (36.19%)
	Presence	7 (21.88%)	25(78.13%)	32 (84.21%)
	Absence	3 (50%)	3 (50%)	6 (15.79%)

MAP, *Mycobacterium avium* subspecies *paratuberculosis*; PTB, paratuberculosis; nV, non-vaccinated; V, vaccinated; PCR, polymerase chain reaction; ZN, Ziehl-Neelsen staining; IHC, immunohistochemistry; (+), positive; (-), negative.

lesions in MS LNs and/or the ICV. The results are summarized in Table 3.

4 Discussion

The direct and indirect economic losses derived from PTB presence in bovine, ovine, and caprine herds have been described worldwide (10, 22, 26, 38, 39). However, diagnostic test sensitivity in the early subclinical stages of the disease is low, and further investigation is needed to address this knowledge gap (6, 22, 40, 41).

CIT tests are widely used in control programs for TB, although their sensitivity is low, and cross-reactions have been described (8, 10, 41, 42). In the present study, a comparative CIT test was used to reduce the risk of non-specific reactions caused by other non-tuberculous mycobacteria (41). Furthermore, although false-positive results originating from the anti-MAP vaccine have been previously reported, no differences were observed between

the V and nV CIT-positive animals in our study (8, 10, 41, 42). Nevertheless, statistically, more nV goats with PTB-compatible gross and histopathological lesions presented inconclusive results for aPPD, which confirms the importance of the implementation of the CIT test and the need for subsequent morphological assessment for correct herd diagnosis.

It is worth highlighting that the gross lesions described in this study are not specific to PTB and are not always present in infected animals (5, 6, 11, 12, 24–26). Nevertheless, PTB-compatible gross lesions were mostly detected in nV animals in this study. This might suggest the effect of anti-MAP vaccination in the reduction of PTB lesions and its previously described heterogeneous protective effect in vaccinated herds (8, 11, 14, 17, 43, 44).

In the present study, a considerable percentage of the PTB-compatible lesions could only be detected histologically. Our results highlight the importance of microscopic examination of target organs, as previously stated by other authors (25, 27, 44–46). Furthermore, we used two grading systems to describe the lesions in the MS LNs and the ICV. Goats are particularly susceptible to both PTB and TB, with calcified granulomas being frequently reported, suggesting a limited ability to control the infection (12, 25, 47). Thus, a grading system distinguishing between 4 granuloma stages was applied in this study to classify the severity and the chronic onset of the lesions of MS LNs independently from the amount of mycobacteria present (36).

The MS LNs presented granulomatous lymphadenitis in more nV goats than V goats. Although no such association was demonstrated regarding the grading of the lesions, it is worth pointing out that stage IV granulomas with central necrosis and mineralization were detected in 60% of the affected animals, of which 70% were nV, and only 30% were V. Similar lesions had been frequently reported in caprine PTB in contrast with bovine PTB (12, 24, 45). Our results suggest a possible relationship between the reduction of granulomatous lymphadenitis affecting MS LNs and anti-MAP vaccination. Additionally, the importance of histopathological assessment is confirmed as granulomas can only be grossly detected when they are mineralized (grades III and IV), and those lesions were found to a lesser extent in the V animals. Similar results have been previously reported in both experimental and natural infections, although the histopathological lesions in MS LNs in subclinical cases have not been graded specifically (10, 11, 14, 15, 17, 43).

The grading system applied in this study to evaluate the ICV was chosen as it relies on the morphological description of the lesions detected with HE stain in terms of severity and distribution (23). On the contrary, the widely used score proposed by Corpa et al. is centered on the distribution, the subjectively evaluated intensity, the predominant cell type detected in the lesions, as well as the bacterial load detected by ZN (12). Furthermore, a modification to the initial grading system was implemented, and the LP and the PPs were evaluated separately, as various authors have previously reported differences between the lesions in those two sites in the initial stages of the disease (12, 24, 27). The PPs presented granulomatous lesions in twice as many cases as the LPs. A previous study in naturally infected goats reports that in cases with focal lesions, granulomatous infiltrate is more common in the PPs than in the LP, although not all cases described were subclinical (12). On the other hand, an experimental study focused on the gut-associated lymphoid tissue of PTB cases reported lesions affecting the PPs in 6/7, and it examined 2-year-old animals with no clinical signs (48).

Regarding the vaccination status, statistical differences were demonstrated between the V and the nV animals in relation to the PTB-compatible lesions in both LP and the PPs, with those being classified as mild and multifocal in most cases. Various authors demonstrate the benefit of anti-MAP vaccination on the reduction of losses due to PTB in affected herds (10, 11, 14, 15, 17, 43). In a recent study evaluating the effect of anti-MAP vaccination, only one animal with multifocal lesions affecting both LP and PPs was found, and it was nV, although the authors did not use the same classification as the one applied in our study and conducted the on-field study in a herd with a low prevalence of MAP infection (14). On the contrary, another study conducted in naturally infected goats shows a reduction in the grade of MAP-induced lesions in the target organs of V animals, grading those as mild/multifocal, although not distinguishing between lesion sites (8). In relation to the age of the affected animals, a statistical difference was only demonstrated in the nV group, in which most of the animals with PTB-compatible lesions were between 12 and 24 months of age. Those results must be interpreted with caution as the study was conducted under natural infection conditions, and thus, we have no data about the exact time of infection or the dose of MAP. It is worth highlighting that clinical disease in goat PTB usually appears earlier than in cows (23, 49), although the data about the age of appearance of histopathological lesions is limited. One experimental study found PTB-compatible lesions in goat kids as soon as 3 months post-infection, and those were more severe than in animals evaluated 6 and 12 months post-infection (23). Another experimental study reports lesions in target organs found in subclinical cases 2 years after inoculation (48). In natural infection, clinical and subclinical cases have been reported in animals between 1.5 and 8 years of age (12). The results of our study confirm the chronic onset of the disease in the ICV, although further studies are needed to analyze the effect of vaccination on the age of PTB lesions development.

The identification of MAP in target organs was performed by ZN, IHC, and PCR, and more nV goats than V goats were identified as positive. In the case of the ZN, AFB was detected in the MS LNs and, to a lesser extent, in the ICV, although it is worth mentioning that 13.64% of all ZN-positive animals only presented AFB in the ICV and were nV. The ICV has been described as the only organ histologically affected in the early subclinical stages of goat PTB (11, 12, 24, 27), and our results highlight the importance of its examination for accurate postmortem diagnosis. The ZN negativity in animals with histopathological lesions can be partially explained by the fact that AFB is only stained by ZN when they are intact, as previously reported in both clinical and subclinical cases (28, 50). Furthermore, the differences observed between the V goats and the nV goats in terms of both positivity and amount of AFB might indicate the benefit of vaccination on the reduction of MAP load in target organs. This result can be related to previous studies that reported a decrease in MAP shedding in V herds with both high and low prevalences of MAP infection (10, 11, 17, 43).

The results of the IHC were in line with the ZN ones, detecting statistically more nV animals as positive. The moderate agreement value obtained in our study might be because IHC can detect MAP antigens in ruptured and dead cells, which have been reported in some forms of PTB and other mycobacterial

infections due to strong cell-mediated host immunity (3, 12, 50–53). Furthermore, the antibody used in this study is polyclonal, and thus, an unspecific reaction cannot be excluded as previously reported by other authors, which might explain the three cases where IHC positivity was detected in the ICV where no PTB-compatible lesions were seen (12, 50–53). A previous experimental study in sheep inoculated with MAP reported limited detection capacity in focal lesions by both ZN and IHC, although the agreement between the two techniques was not analyzed (3). In the case of goat PTB, some authors report substantial agreement between the two techniques in naturally infected herds, although all of them describe that the cases in which ZN and IHC results disagree presented mild/focal lesions with a small amount of MAP (12, 50, 52).

Finally, real-time PCR agreed with the IHC and ZN staining results in 63.81% of the cases. Regarding the cases where PCR was the only technique that identified the presence of PTB, it is worth mentioning that various studies report its high sensitivity and capacity for the detection of small amounts of DNA, even in cases in which no lesions were detected (3, 5, 8, 54, 55). Nevertheless, the extraction process involves a small tissue sample, in which MAP might be absent in cases with a low bacterial load. Conversely, the histological sections used for both IHC and ZN allow the examination of a larger area, potentially explaining why these methods yielded positive results in 4.76% of the animals that were PCR-negative yet exhibited granulomatous lesions (8, 12, 21, 22, 26, 54). The likelihood of false-negative PCR results due to the absence of the amplified sequence is low in this study, as the IS900 sequence is highly conserved among MAP isolates, and all tissues analyzed were fresh and not formalin-fixed (54, 56). Additionally, despite the general agreement among the three diagnostic techniques, a significant difference was observed between the nV and V groups in terms of MAP positivity, aligning with histopathological findings. These results underscore the beneficial impact of vaccination on reducing bacterial load (10, 11, 17, 43) and demonstrate the importance of using a combination of diagnostic tools to maximize the accuracy of PTB detection in subclinical cases. Nevertheless, further studies are needed to optimize sampling protocols, enhance diagnostic agreement, and thereby improve early detection of MAP infection in goats with PTB.

In conclusion, our study quantifies histopathological lesions in natural cases of goat PTB and highlights the importance of both gross and microscopic examination for a correct postmortem diagnosis. Furthermore, histopathological lesions in both MS LNs and ICV were graded in terms of severity and distribution, demonstrating significant differences between V and nV animals. These results might suggest the potential effect of PTB vaccination on the reduction of histopathological lesions in both MS LNs and ICV in subclinical PTB cases. Moreover, we evaluated the agreement among three laboratory techniques—ZN, IHC, and PCR—for the etiological diagnosis of subclinical PTB cases. Our results demonstrate the crucial role of combining these diagnostic tools to assess subclinical PTB in both vaccinated and non-vaccinated goats accurately.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The requirement of ethical approval was waived by Comité Ético de Experimentación Animal (CEEA-ULPGC), Universidad de Las Palmas de Gran Canaria, Spain for the studies involving animals because according to article 2 of RD 53/2013, non-experimental clinical veterinary practices are excluded from the scope of this Royal Decree, and therefore it does not require approval by an Ethics Committee for Animal Experimentation. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

EPS: Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. ES: Investigation, Methodology, Supervision, Validation, Writing – review & editing. AF: Funding acquisition, Resources, Supervision, Writing – review & editing. OQ-C: Investigation, Methodology, Supervision, Writing – original draft, Conceptualization, Funding acquisition. YP-S: Data curation, Investigation, Methodology, Project administration, Supervision, Writing – original draft. AC-R: Methodology, Writing – review & editing. AE: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. PH: Conceptualization, Methodology, Supervision, Writing – review & editing. LD: Conceptualization, Formal analysis, Investigation, Supervision, Writing – review & editing. JB: Conceptualization, Formal analysis, Investigation, Supervision, Writing – review & editing. MP-S: Formal analysis, Investigation, Supervision, Writing – review & editing. IM: Formal analysis, Investigation, Supervision, Writing – review & editing. MR: Formal analysis, Investigation, Supervision, Writing – review & editing. MA: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by CajaCanarias Foundation and “la Caixa” Foundation, grant reference number FCC-FC-2019-03 and by the PROID2020010047 grant from the Canary Regional Ministry of Economy, Knowledge and Work through the Canarian Agency for Research, Innovation and Information Society (ACIISI), for the implementation of I+D projects in the Canary RIS-3 priority areas,

co-financed through PO FEDER Canarias 2014-2020. EPS was the recipient of a predoctoral contract of the Ministry of Universities, Spain (FPU20/03693) and Charter100 2021 scholarship for PhD students' mobility.

Acknowledgments

The authors thank the veterinarians from the Livestock Health Protection Groups responsible for the management of the farms for providing the animals for the study, postmortem technician Javier Ramos, the laboratory technicians of the Institute of Animal Health and Food Safety (IUSA) Ana Afonso Almeda and Alba María Santana Zerpa for their technical assistance, the staff of the Instituto de Ganadería de Montaña (CSIC-ULE), Grulleros, Spain, and the technicians Marta Silva Samos and María Teresa Carro García for the knowledge exchange and laboratory training.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Public Health

TYPE Case Report
PUBLISHED 24 September 2024
DOI 10.3389/fpubh.2024.1387722

OPEN ACCESS

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RECEIVED 18 February 2024
ACCEPTED 29 August 2024
PUBLISHED 24 September 2024

CITATION
Sun Y, Zhang C, Lu B, Chen J and Pan X (2024) Case report: *Mycobacterium chimaera*-induced lymph node infection in a patient with chronic myeloproliferative neoplasm misdiagnosed as tuberculous lymphadenitis.
Front. Public Health 12:1387722.
doi: 10.3389/fpubh.2024.1387722

Case report: *Mycobacterium chimaera*-induced lymph node infection in a patient with chronic myeloproliferative neoplasm misdiagnosed as tuberculous lymphadenitis

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Herein, we report a case of lymphadenitis caused by *Mycobacterium chimaera*. A 54-year-old woman with chronic myeloproliferative neoplasm was admitted to the hospital with cervical lymphadenopathy. After preliminary exclusion of various diseases such as lymphoma, Epstein–Barr virus infection, and autoimmune disease, a lymph node biopsy specimen showed epithelioid granulomatous lymphadenitis with caseous necrosis, epithelial-like cells, and multinucleated giant cells as seen in tuberculosis (TB). Although *Mycobacterium tuberculosis* was never isolated, diagnostic anti-TB treatment was commenced. Following over 9 months of treatment, there was no significant reduction in the size of her cervical lymph nodes, and she continued to experience recurrent low-grade fevers. One sample from the fourth lymph node biopsy tested negative for metagenomic next-generation sequencing (mNGS), and another sample tested positive in the BACTEC MGIT960 liquid culture system, identifying the strains as *Mycobacterium chimaera*. Anti-non-tuberculous mycobacteria (NTM) therapy was initiated, and the patient achieved symptom improvement. In conclusion, NTM lymphoid infection is easily misdiagnosed as long-term etiologic negativity.

KEYWORDS

non-tuberculous mycobacteria, *Mycobacterium avium* complex, *Mycobacterium chimaera*, lymphadenitis, diagnosis, case report

1 Introduction

Non-tuberculous mycobacteria (NTM) refer to mycobacteria other than *Mycobacterium leprae* and *Mycobacterium tuberculosis* complex. Over the past few decades, NTM epidemics have increased globally (1–3). As NTM closely resembles *Mycobacterium tuberculosis* in pathogenesis, clinical manifestations, and pathology, NTM infection, particularly extrapulmonary NTM infection, tends to be atypical and highly uncommon. Consequently, its diagnosis is often challenging and delayed (4, 5). *Mycobacterium avium* complex is a major

NTM associated with infections (3), and *Mycobacterium chimaera* (MC) is a member of the *Mycobacterium avium* complex. Lymphadenitis caused by *Mycobacterium avium* complex or another NTM is more common in children than in adults (6), and extrapulmonary NTM infections in adults usually occur in individuals with congenital or acquired immunodeficiency (7). Herein, we present a case of lymph node infection caused by MC. Four lymph node biopsies and extensive laboratory, imaging, and pathological examinations were performed. MC was obtained by lymph node culture, and symptom improvement was achieved. The entire process of diagnosis and treatment may provide a reference for the clinical diagnosis and treatment of similar patients. This case study can improve clinicians' understanding of NTM-related diseases, reduce misdiagnoses and missed diagnoses, and avoid delays in treatment.

2 Case description

On 31 March 2022, a 54-year-old woman was admitted to our hospital after finding a mass in the right cervical root 4 days prior to admission. She had a 6-year history of thrombocytosis and had no other relevant medical or surgical history. Physical examination revealed a hard, fixed mass approximately 3 cm in diameter in the right cervical lymph node, with indistinct boundaries and no tenderness. The patient did not exhibit symptoms such as chills, fever, cough, expectoration, weight loss, or night sweats. To elucidate the nature of the lymphadenopathy, the following tests were conducted.

2.1 Laboratory tests

Blood cytology showed an elevated platelet count (825×10^9/L), white blood cell count within normal range (7.06×10^9/L), reduced absolute Lymphocytes count (821×10^6/L), and reduced differential lymphocyte count (T Lymphocytes: 799×10^6/L, $CD3^+CD4^+$ T cells: 456×10^6/L, $CD3^+CD8^+$ T cells: 308×10^6/L, B Lymphocytes: 9×10^6/L, and NK cells: 8×10^6/L). An Epstein-Barr virus (EBV) test revealed evidence of a previous infection with 6.77×10^2 DNA copies/ml of a whole blood sample (↑400), EBV capsid antigen IgG antibody titer >750 U/mL, and EBV nuclear antigen IgG antibody titer >600 U/mL; EBV capsid antigen IgM antibody was normal. Moreover, neurogenic specific enolase was 25.48 ng/mL (↑ < 16.3), cytokeratin 19 was 5.85 ng/mL (↑ < 3.3), and uric acid was 495 μmol/L (↑155–357). Anti-nuclear antibody was positive at 1:100 nuclear granule of the main karyotype, and rheumatoid factor was 26 IU/mL (↑ < 20), while tests for anti-Streptolysin "O," anti-cyclic citrullinated peptide antibody, anti-neutrophil cytoplasmic antibody, immunoglobulin G4, and anticardiolipin antibody were all negative. The T-SPOT test was non-reactive. Serological tests for erythrocyte sedimentation rate, C-reactive protein, procalcitonin, human immunodeficiency virus, hepatitis B, hepatitis C, and syphilis tests were normal. Additionally, her immunoglobulin A, M, G, complement C3, C4, interleukin-2 (IL-2), IL-4, IL-6, IL-10, interferon-γ, and tumor necrosis factor levels were within the normal range. Laboratory parameters on admission are presented in Table 1. Abnormal myeloid precursor cells were detected in her bone marrow morphology and flow cytometry, suggesting a link with myeloproliferative neoplasm. According to the

TABLE 1 Patient's laboratory parameters on first and second admissions to Affiliated Dongyang Hospital of Wenzhou Medical University.

Parameter	Value	Reference range
First admission		
White blood cell count [10^9/L]	7.06	3.5–9.5
Lymphocytes [10^6/L]	821↓	1,459–2,633
T Lymphocytes ($CD3^+$) [10^6/L]	799↓	919–1817
$CD3^+CD4^+$ T cells [10^6/L]	456↓	467–949
$CD3^+CD8^+$ T cells [10^6/L]	308	292–830
B Lymphocytes [10^6/L]	9↓	107–319
NK cells [10^6/L]	8↓	162–590
Platelets [10^9/L]	825↑	125–350
Epstein–Barr virus DNA [copies/ml]	677↑	400
Epstein–Barr virus capsid antigen IgG antibody[U/ml]	>750↑	<20
Epstein–Barr virus nuclear antigen IgG antibody [U/ml]	>600↑	<20
Epstein–Barr virus capsid antigen IgM antibody [U/ml]	0.78	<40
Procalcitonin [ng/ml]	0.085	<0.1
C-reactive protein (serum) [mg/L]	3.4	<5
Serological tests for erythrocyte sedimentation rate [mm/h]	29	<38
Neurogenic-specific enolase [ng/ml]	25.48↑	<16.3
Cytokeratin 19 [ng/ml]	5.85↑	<3.3
IgG4 [g/L]	0.82	<2.01
IgG [g/L]	14.8	7–16
IgA [g/L]	1.80	0.70–4.00
IgM [g/L]	0.62	0.40–2.30
Complement C3[g/L]	1.14	0.80–1.60
Complement C4[g/L]	0.18	0.10–0.50
IL-2 [pg/ml]	2.58	<7.5
IL-4 [pg/ml]	<2.44	<8.56
IL-6 [pg/ml]	4.01	<5.4
IL-10 [pg/ml]	2.98	<12.9
Interferon-γ [pg/ml]	18.25	<23.1
Tumor necrosis factor [pg/ml]	<2.44	<16.5
Uric acid [μmol/l]	495↑	155–357
Rheumatoid factor [IU/ml]	26↑	<20
Anti-streptolysin "O" [IU/ml]	<20	<160
Anti-cyclic citrullinated peptide antibody [IU/ml]	<8	<17
Anticardiolipin antibody	Neg.	
Anti-nuclear antibody	Positive at 1:100 nuclear granule of the main karyotype	

(Continued)

TABLE 1 (Continued)

Parameter	Value	Reference range
Anti-neutrophil cytoplasmic antibody	Neg.	
Hepatitis B surface antigen	Neg.	
Hepatitis B surface antibody	Neg.	
Hepatitis B e antigen	Neg.	
Hepatitis B e antibody	Neg.	
Hepatitis B core antibody	Neg.	
Treponema pallidum antibody	Neg.	
Human immunodeficiency virus antibody/p24 antigen	Neg.	
Hepatitis C virus antibody	Neg.	
T-SPOT	Neg.	
Second admission		
White blood cell count [10^9/L]	7.64	3.5–9.5
Lymphocytes [10^6/L]	788↓	1,459–2,633
T Lymphocytes ($CD3^+$) [10^6/L]	739↓	919–1817
$CD3^+CD4^+$ T cells [10^6/L]	405↓	467–949
$CD3^+CD8^+$ T cells [10^6/L]	300	292–830
B Lymphocytes [10^6/L]	6↓	107–319
NK cells [10^6/L]	27↓	162–590
Platelets [10^9/L]	560↑	125–350
Epstein–Barr virus DNA [copies/ml]	1930↑	400
Epstein–Barr virus capsid antigen IgG antibody [U/ml]	>750↑	<20
Epstein–Barr virus nuclear antigen IgG antibody [U/ml]	>600↑	<20
Epstein–Barr virus capsid antigen IgM antibody [U/ml]	0.77	<40
Uric acid [μmol/l]	495↑	155–357
rheumatoid factor [IU/ml]	24↑	<20
Anti-nuclear antibody	Neg.	
T-SPOT	Neg.	

IL, interleukin; Neg., negative.

result of genetic testing, mutations in ASXL1, SETBP1, U2AF1, and PTPN11 were observed, and a diagnosis of chronic myeloproliferative neoplasm-primary thrombocytosis was made. Hydroxyurea and aspirin were administered as anticoagulants and for the treatment of thrombocytosis.

2.2 Medical imaging

Color Doppler ultrasound revealed multiple lymphadenopathies in the bilateral supraclavicular (Figures 1A,B), neck, armpit, and inguinal regions. Plain chest computed tomography (CT) and X-ray images showed clear pulmonary textures in both lungs, and there were no exudates or space-occupying lesions in the lung parenchyma. Enhanced CT revealed scattered infections in both lungs, accompanied by a pleural reaction on both sides. Multiple enlarged lymph nodes were observed in the neck and mediastinum, along with an enlarged spleen and multiple low-density foci within. A cyst was noted in the right kidney, and a small amount of fluid was present in the pelvic cavity. Her whole-body positron emission tomography-CT (PET-CT) examination revealed excessive fluorodeoxyglucose (FDG) accumulation in multiple swollen lymph nodes (bilateral supraclavicular, neck, mediastinum, and left inguinal regions), particularly in the right supraclavicular region, measuring approximately 27 mm in diameter, with increased 18-FDG uptake, having a maximum standard uptake value (SUVmax) of 15.6 (Figure 2A). Excessive accumulation was noted in the mediastinal lymph nodes, with an SUVmax of 14.0. The largest node measured approximately 25 × 13 mm (Figures 2A,B). Additionally, multiple circular low-density foci of varying sizes were observed in the spleen, the largest of which was approximately 30 mm in diameter, with an elevated FDG uptake and SUVmax of 10.8. There was also diffuse FDG uptake in the bone marrow of the axial skeleton and limbs, with an SUVmax of 4.3. Considering the patient's history and atypical symptoms of TB, the likelihood of lymphoma was considered to be high.

2.3 Pathological examination

Two lymph node biopsies were performed during the hospital stay, revealing chronic lymphadenitis with local granuloma formation (Figure 3A), focal necrosis, and acute and chronic inflammatory cell infiltration (Figure 3A). Epithelial-like cells (Figure 3B) and multinucleated giant cells were observed locally, and lymphoid structures were partially absent. Lymphocytes had either a mass or diffuse distribution. Neutrophils and plasma cells were also observed, and there was evidence of proliferation of fibrous tissue. The results of immunohistochemical staining were as follows: acid-fast staining (−), periodic acid-Schiff staining (−), Alcian blue-periodic acid-Shiff (−), S-100 (−), CD3 (t-cell +), Cd5 (t-cell +), CD20 (b-cell +), CD79a (B-cell +), CD1a (−), CD68 (histiocytes +), and Myeloperoxidase (neutrophil +). No malignant cells were found in the liquid-based cytology of the left supraclavicular lymph node. At the same time, flow cytometry of the biopsies showed no lymphoma cells.

Before further examination to confirm the diagnosis, the patient voluntarily transferred to the First Affiliated Hospital of Zhejiang University. On 13 May 2022, biopsies were performed on the left inguinal and right cervical lymph nodes. The report showed negative results for the right cervical lymph nodes: Xpert MTB/RIF assay (Cepheid), periodic acid-Schiff staining, and fungus (Fluorescence *in situ* hybridization). Histopathological analysis of the right cervical lymph node revealed granulomatous inflammation with focal caseous necrosis. The left inguinal lymph nodes exhibited reactive hyperplasia and were treated with diagnostic anti-TB treatment for lack of etiological evidence. The patient commenced treatment with isoniazid, rifampicin, ethambutol, and pyrazinamide on 15 May 2022. On 15 July 2022, CT scans of the chest and abdomen revealed multiple enlarged lymph nodes in the right clavicle and mediastinum post-anti-TB treatment, indicating a reduction in size compared to that of previous scans. Infection was observed in both lungs, showing signs of absorption and scattered proliferative foci. On 28 July 2022, isoniazid, rifampicin, and ethambutol were continued as anti-TB treatments.

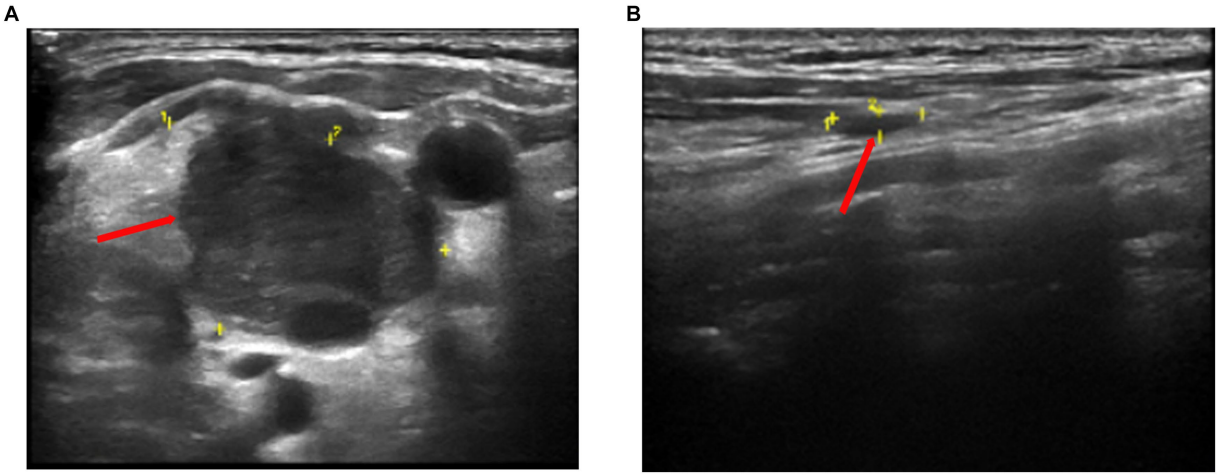

FIGURE 1
Color doppler sonography showed multiple echogenic lymph nodes of different sizes on both clavicles. **(A)** The larger one on the right is approximately 28 mm × 20 mm in size (yellow section and red arrow). **(B)** The larger one on the left is approximately 25 mm × 8 mm in size (yellow section and red arrow).

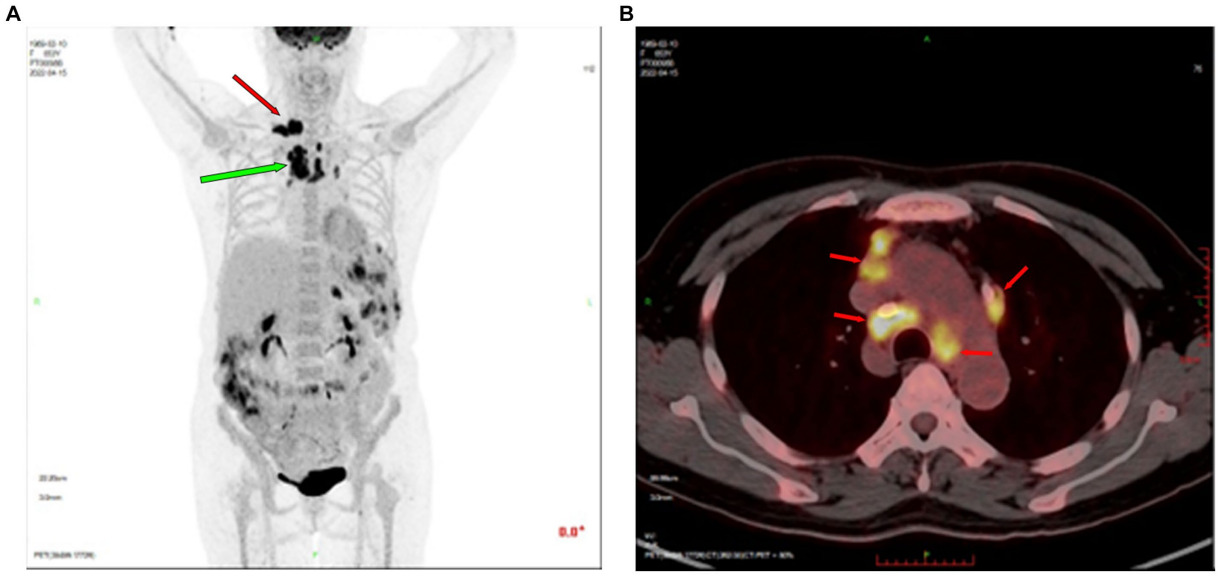

FIGURE 2
Positron emission tomography-CT revealed excessive FDG accumulation in multiple lymph nodes. **(A)** The right supraclavicular lymph node, approximately 27 mm in diameter, showed increased FDG uptake (SUVmax = 15.6, indicated by red arrow). Enlarged mediastinal lymph nodes showed increased FDG uptake (green arrow). **(B)** Excessive FDG accumulation in mediastinal lymph nodes (red arrow). CT, computed tomography; FDG, fluorodeoxyglucose; SUVmax, maximum standard uptake value.

On 31 January 2023, she was admitted to our hospital for a second time because of dizziness and chest tightness for 3 days. Despite undergoing more than 9 months of anti-TB therapy, the patient showed little improvement, and there were recurring symptoms accompanied by a low-grade fever. During hospitalization, the purified protein derivative test was strongly positive, and the T-SPOT was negative. Blood cytology showed a decrease in platelet count when compared to the first admission (560×10^9/L), while the absolute counts of total lymphocytes and multiple subpopulations were still low (Table 1). EBV were 1930 DNA copies/ml of a whole blood sample and EBV capsid antigen IgM antibody was normal. Anti-nuclear antibody was negative (Table 1). To confirm the cause of lymphadenopathy, a subsequent biopsy was performed. The Xpert MTB/RIF assay (Cepheid) yielded a negative result on the left supraclavicular lymph node biopsy, and metagenomic next-generation sequencing (mNGS) did not detect any pathogenic microorganisms in the tissues. Another sample was inoculated in MGIT960 for 11 days, where acid-fast bacteria were detected (Figure 3C). The strain was identified as MC by sequencing heat shock proteins 65 (8) on 21 February 2023. Following a positive result in the tube, the tissue initially sent for

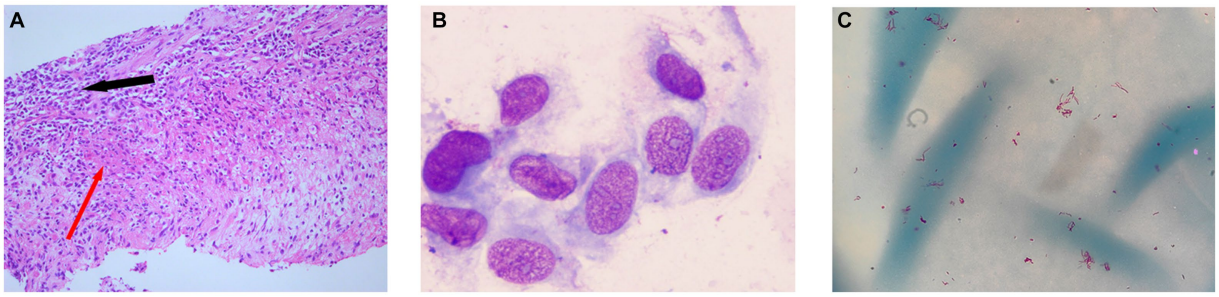

FIGURE 3
(A) Lymph node biopsy: granulomatous inflammation (red arrow) and acute and chronic inflammatory cell infiltration (black arrow). **(B)** Epithelial-like cells by Wright–Giemsa stain. **(C)** Acid-fast bacteria (red color) were isolated from the MGIT960 liquid culture tube after centrifugation concentration and acid-fast staining.

mNGS was also inoculated in MGIT960 but remained negative after 6 weeks. We inferred the presence of an NTM infection based on (1) low-grade fever, (2) high uptake of PET-CT in patients with inflammation and NTM infection, (3) histopathological examination, and (4) positive culture results of MGIT960. As the patient had been discharged from the hospital, we called her with the news that MC had been detected and suggested that she come to the hospital for treatment on 25 March 2023.

For personal reasons, she postponed returning to our hospital until 7 May 2023. At this point, we initiated anti-NTM treatment with azithromycin, linezolid, rifampicin, and moxifloxacin, and her condition improved. After discharge from the hospital, she stopped the medicines on 6 June 2023, owing to physical intolerance. On 8 August 2023, she was hospitalized again for pain and swelling of the right cervical lymph nodes and was treated with daily moxifloxacin, rifampicin, azithromycin, and doxycycline. At her follow-up after more than 6 months of taking these medications, no tenderness was observed in the right cervical lymph nodes, the swelling had decreased, and the absolute lymphocyte count (760×10^6/L) was still below the lower limit of normal, platelet count was 380×10^9/L. After 11 months of continuous medication, an enhanced CT scan was performed on 10 July 2024, which revealed that the low-density lesions in the spleen were fewer and smaller than previously observed, with the largest lesion measuring approximately 7 mm in its longest dimension. Color Doppler ultrasound revealed that the cervical lymph nodes were much smaller than before, showing that the treatment had been effective.

3 Discussion

Lymphadenopathy is a common clinical symptom caused by the proliferation of cells in the lymph nodes or infiltration of tumor cells. Notably, it can occur in several diseases. Our patient's lymphadenopathy involved multiple parts of the body. EBV is closely associated with some types of lymphoma. Our patient's EBV DNA levels were slightly elevated, and the splenic changes along with abnormally high uptake on PET-CT raised suspicions of lymphoma. Moreover, the atypical nature of the infection initially posed challenges for differential diagnosis. Furthermore, as the patient's swollen lymph nodes were located near blood vessels and nerves, the tissue samples could only be obtained through fine needle aspiration, which is inherently limited. Moreover, the detection of positive bacteria in only one tube of liquid medium suggests that low bacterial counts may also present challenges for laboratory testing. It is recommended that patients who are unable to undergo a surgical excision biopsy should be considered for multipoint puncture with a core needle. In conjunction with mNGS, various molecular biology techniques, as well as solid and liquid TB cultures, can improve the detection rate (9–11). Furthermore, some studies indicate that in cases where NTM disease is suspected, avoidance of certain antibiotics during specimen collection is advised, specifically macrolides, quinolones, aminoglycosides, sulfamethoxazole-trimethoprim, and linezolid. Ideally, samples should be collected at least 2 weeks after the discontinuation of these medications, if necessary (12, 13). This is difficult to perform clinically; however, in patients with mild disease, as many specimens as possible are collected after discontinuation to increase the detection sensitivity of NTM.

The NTM widely exists in water, soil, dust, and other natural environments. Planting soil, hospital water, and even shower heads can be rich in *Mycobacterium avium* complex. Human exposure to NTM is common, but epidemics of NTM infection are fairly infrequent, suggesting that NTM has low-to-moderate pathogenicity and that host risk factors play an integral role in vulnerability to NTM disease (14). Individuals with immunodeficiencies or underlying diseases are susceptible to NTM infection. The patient is a menopausal female, living in the city and not involved in farm work. She had no history of acupuncture, surgery, or use of immunosuppressants. We suggested that the ongoing reduction in the counts of T, B, and natural killer cells in the peripheral blood might be associated with her disease. Innate and adaptive immune cells are known to play significant roles in the host's resistance to NTM infection and are associated with the exacerbation and progression of the disease (15, 16).

Patients receiving medication outside the hospital, without full supervision by a doctor, often do not undergo a further investigation into the causes of ineffective treatment, leading to prolonged misdiagnosis. Our patient discontinued her anti-NTM therapy, owing to physical intolerance, without consulting with a doctor. Therefore, we recommend that clinicians closely monitor patients with mycobacterial infections enrolled in treatment programs and gather clinical, imaging, and microbiological data. This approach is essential to evaluating treatment response and adherence. Timely identification of adverse drug reactions enhances the likelihood of successful treatment completion (17).

4 Conclusion

The NTM infections in lymph nodes can often be misdiagnosed in the absence of bacterial identification. In instances of prolonged etiological negativity, an experienced multidisciplinary team undertakes a comprehensive evaluation of all potential diagnoses. Clinicians should be alert to patients with long-term lymphocyte cell reduction. In such cases, employing rational and standardized sampling, along with a combination of various detection methods, is crucial for the effective diagnosis, management, and treatment of patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

This study was approved by the Ethics Committee of Affiliated Dongyang Hospital of Wenzhou Medical University (approval no.: 2024-YX-008). Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

YS: Data curation, Methodology, Writing – original draft, Writing – review & editing. CZ: Methodology, Validation, Writing – original draft. BL: Investigation, Resources, Writing – review & editing. JC: Investigation, Resources, Writing – review & editing. XP: Conceptualization, Methodology, Software, Visualization, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors thank the patient involved in this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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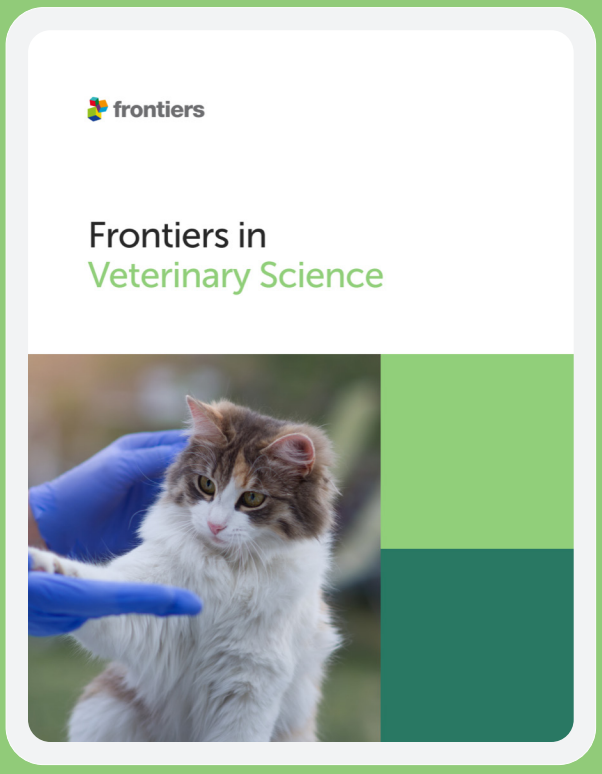

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