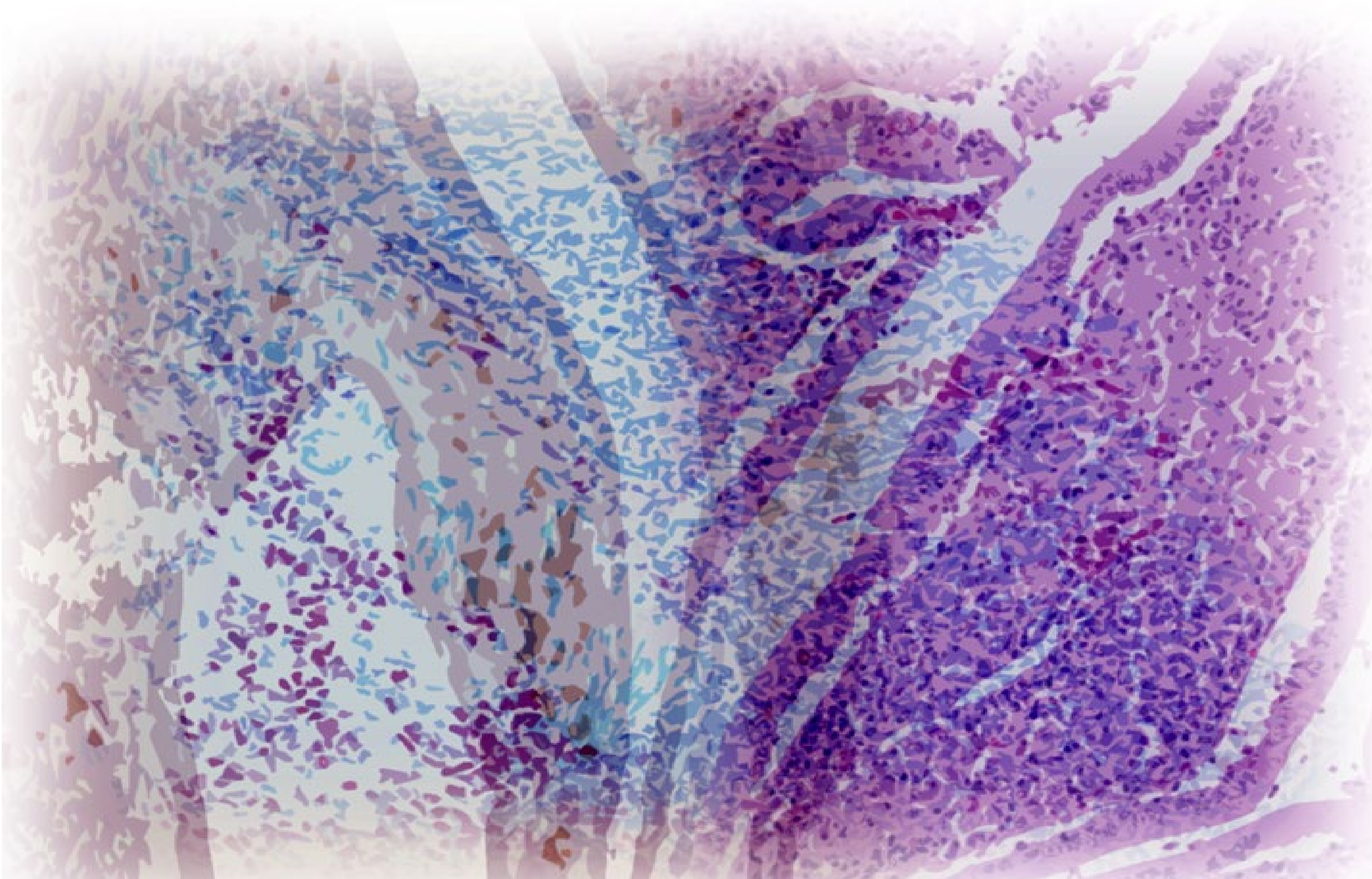
ULPGC
Universidad de
Las Palmas de
Gran Canaria

IUSA
Instituto Universitario de Sanidad Animal
y Seguridad Alimentaria

ESTUDIO ETIOPATOGÉNICO DE LA PARATUBERCULOSIS CAPRINA EN CANARIAS



Elena Plamenova Stefanova

Doctorado en Sanidad Animal y Seguridad Alimentaria

Arucas, septiembre 2024

ULPGC Universidad de Las Palmas de Gran Canaria | IUSA Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

Tesis doctoral

ESTUDIO ETIOPATOGÉNICO DE LA PARATUBERCULOSIS CAPRINA EN CANARIAS

Elena Plamenova Stefanova

Doctorado en Sanidad Animal y Seguridad Alimentaria

Escuela de Doctorado - Universidad de Las Palmas de Gran Canaria

Arucas, septiembre 2024

ULPGC Universidad de Las Palmas de Gran Canaria | Escuela de Doctorado

D. ANTONIO J. FERNÁNDEZ RODRÍGUEZ, COORDINADOR DEL PROGRAMA DE DOCTORADO DE SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

INFORMA,

De que la Comisión Académica del Programa de Doctorado, en su sesión de fecha …./…./……, tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada **"Estudio etiopatogénico de la paratuberculosis caprina en Canarias"** presentada por la doctoranda Dª **Elena Plamenova Stefanova** y dirigida por Dra. Dña. **Marisa Ana Andrada Borzollino**, Dr. D. **I. Óscar Quesada Canales**, y Dr. D. **Antonio J. Fernández Rodríguez**.

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 04/03/2019) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Arucas, a … de ……. de dos mil veinticuatro.

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

ESCUELA DE DOCTORADO

Programa de doctorado

DOCTORADO EN SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA

Título de la Tesis

ESTUDIO ETIOPATOGÉNICO DE LA PARATUBERCULOSIS CAPRINA EN CANARIAS

Tesis Doctoral presentada por Dña.

ELENA PLAMENOVA STEFANOVA

Dirigida por

Dra. Dña. MARISA ANA ANDRADA BORZOLLINO

Dr. D. I. ÓSCAR QUESADA CANALES

Dr. D. ANTONIO J. FERNÁNDEZ RODRÍGUEZ

Arucas, a ..... de ....de 2024

Los Directores La Doctoranda

A mis padres y a mi hermano

A mis abuelos

A mi familia

A Óscar

A la memoria de Valentín Pérez

“It is always darkest before the dawn”

Thomas Fuller, 1650

Stefan Stefanov, my brother, 2021

INDICE

1. ABSTRACT

Introduction

Paratuberculosis (PTB), also known as Johne's disease, is a chronic, contagious disease primarily affecting domestic and wild ruminants. It is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The disease is particularly challenging due to its slow development and subclinical stages, during which infected animals shed the bacteria, contaminating the environment. The disease is of significant concern in animal health due to its economic impact on livestock, especially ruminants, as it leads to reduced milk production, weight loss, and death.

The Canary Islands play an essential role in Spain's goat farming industry, holding the fourth-largest population of goats in the country. The region has been officially recognized as free from bovine tuberculosis (TB) caused by *Mycobacterium tuberculosis* complex since 2017, a status of critical importance in maintaining the health of livestock and ensuring the economic viability of farming activities. However, managing PTB presents unique challenges, particularly because the anti-MAP vaccination can interfere with the official diagnostic tests for TB. As a result, goat farms in the Canary Islands are required to obtain special authorization before vaccinating against MAP. This authorization process includes certifying that the farm is free from TB and confirming the presence of PTB on the farm. Once these conditions are met, farmers may apply for vaccination permits in line with local regulations (Decree 51/2018).

This doctoral research focuses on caprine PTB in the Canary Islands. The aim is to evaluate the disease pathology, vaccination effects, and prevalence using a combination of diagnostic techniques, providing information to improve disease diagnosis and control strategies in a privileged health context, the condition of insularity, and productive systems.

Objectives

The aims of this work are:

1. Evaluate the morphological lesions associated with clinical and subclinical paratuberculosis in naturally infected goat herds.

2. Recognize the different concomitant pathological findings in goats naturally infected with PTB
3. Implement a more effective diagnosis for identifying MAP using immunohistochemical and molecular biology techniques.
4. Study the impact of anti-MAP vaccination on etiological identification, lesion severity and distribution in clinical and subclinical cases of natural infection with caprine PTB.
5. Determine the prevalence of paratuberculosis in goat herds across the Canary Islands, Spain applying serological assay. Evaluate the immune response in relation to age, environment and farm characteristics.
6. Analyze immune responses to anti-MAP vaccination in relation to age in PTB naturally infected herds on the Canary Islands, Spain.

Summary of Publication I: Morphological Assessment of Concomitant Lesions in Goat Herds Infected with Paratuberculosis

This study investigated the lesions associated with MAP infections in vaccinated and non-vaccinated goats. The primary goal was to assess the extent and nature of the pathological changes induced by MAP and any concomitant lesions in naturally infected animals. The study involved 39 goats (15 vaccinated and 24 non-vaccinated) with PTB-compatible histological lesions from various farms with confirmed history of disease. Samples were collected at necropsy and processed routinely for histopathological evaluation. Granulomatous lesions in mesenteric lymph nodes (MS LNs) were classified from grade 0, no lesions to grade IV, necrosis and mineralization. Granulomatous enteritis affecting the ileocecal valve (ICV) was graded separately in lamina propria (LP) and Peyer´s patches (PPs) in terms of severity and distribution. Statistical analysis was performed using IBM SPSS Statistics 27 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA: IBM Corp).

Gross PTB findings were registered affecting the MS LNs in 61.5% and the ICV in 17.9% of predominantly non-vaccinated animals. Granulomatous enteritis in the vaccinated cases was of mild severity in contrast with the non-vaccinated in which lesions varied from mild to severe. The lymphadenitis in the MS LNs in both groups was mainly grade IV with well-formed encapsulated granulomas with central necrosis and mineralization.

The main concomitant pathologies identified in the studied animals were of inflammatory origin involving most commonly the hemolymphatic, respiratory and gastrointestinal systems. More non-vaccinated goats with PTB-compatible enteritis in the ICV presented respiratory lesions (p=0.027). Furthermore, lung inflammation in vaccinated goats was only detected in animals between (12-24] months of age in contrast with the non-vaccinated among which those findings were registered in all age ranges (p=0.035). To the authors knowledge, this is the first work that describes and evaluates the concomitant pathologies in naturally infected caprine herds. The study underscores the importance of histopathological examination in diagnosing and controlling caprine PTB and gives new insights on the potential protective effect of vaccination on both MAP induced lesions and concomitant pathologies present in the herd.

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

This study focused on comparing the diagnostic methods and lesion severity in pre- and post-vaccinated goats across several herds. A total of 105 goats were evaluated at slaughter, with 61 non-vaccinated and 44 vaccinated. Granulomatous lesions in the mesenteric lymph nodes (MS LNs) and ileocecal valve (ICV) lamina propria (LP) and Peyer's Patches (PPs) were evaluated through histopathological analysis. MAP was identified using Ziehl-Neelsen staining (ZN), immunohistochemistry for MAP antigen detection (IHC), and PCR targeting the insertion sequence 900 (IS 900). Statistical analysis was performed using IBM SPSS Statistics 27 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA: IBM Corp).

PTB-compatible gross lesions were detected in 39.00% of the animals, of which 68.29% were non-vaccinated and 31.72% were vaccinated. The inclusion of histopathological tools improved the diagnostic accuracy by 28% in MS LNs and by 86.05% in ICV. The microscopic lesions in MS LNs, ICV LP and ICV PPs were predominantly detected in non-vaccinated animals with the differences being statistically significant with $p<0.05$. Also, the bacterial load detected by ZN, IHC and PCR was lower in the vaccinated animals with $p<0.05$. Nevertheless, the agreement between the three techniques varied from low to moderate.

The results confirm that vaccination reduces the severity and distribution of MAP induced lesions and bacterial load in target organs, although it does not fully prevent infection. Furthermore, the study emphasizes the importance of combining post-mortem examination with different laboratory techniques for agent identification to fully detect, assess and control subclinical caprine PTB infections in affected herds.

Summary of Publication III: Caprine Paratuberculosis Seroprevalence and Immune Response to Vaccination

This study examined the seroprevalence of PTB and the immune response to MAP vaccination in 12 goat farms across the Canary Islands. It involved 2,774 serum samples analyzed through commercial ELISA kit (*Mycobacterium paratuberculosis* Test Kit PARACHEK® 2, Prionics AG, Schlieren, Switzerland) to detect anti-MAP antibodies. Statistical analysis was performed using IBM SPSS Statistics 27 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA: IBM Corp).

The initial apparent seroprevalence was 18.4%, with variations across farms ranging from 2.5% up to 61.1%. Three prevalence groups were established to analyze the differences between the farms: A, (0-10%]; B, (10-20%] and C, >20%. In the first session, significant differences between the farms regardless the age of the animals were only demonstrated in group C ($p=0.001$). Nevertheless, differences between the number and proportion of ELISA positive goats in the different age ranges was confirmed in the three prevalence groups. Also, differences between the antibody development and farm mean annual temperature, fencing and proximity to other farms were demonstrated in group C.

The second phase of the study focused on the immune response to vaccination. In group A, seroconvertion levels were higher among older animals. In groups B and C, the antibody response was heterogeneous. Although, only in group C the differences between the number and proportion of ELISA positive animals in each age groups were statistically significant.

The study highlights the need for ongoing serological monitoring and integrated diagnostic approaches to manage PTB effectively in vaccinated and non-vaccinated goat herds on the Canary Islands.

Conclusions

1. Histopathological examination is important for correct diagnosis of caprine PTB in both clinical and subclinical cases as by gross examination neither granulomatous lymphadenitis with microgranuloma formation, nor mild and multifocal noncalcified granulomatous enteritis can be detected.
2. The most common concomitant pathologies found in goat herds with natural PTB infection on the Canary Islands are of inflammatory origin and mainly affect the hemolymphatic, respiratory and gastrointestinal systems.
3. Vaccination against MAP might have a beneficial effect on the reduction of concomitant pathologies such as respiratory inflammation processes.
4. It is advisable to use a combination of laboratory techniques including Zielh-Neelsen, immunohistochemistry and PCR targeting IS900 for correct etiological identification of MAP in naturally infected goats, as in cases with low bacterial load the agreement between the three techniques varies from low to moderate.
5. Vaccination against MAP can mitigate the severity of the granulomatous enteritis to mild multifocal lesions and reduce the bacterial load in target organs but cannot fully prevent infections. Mesenteric lymph nodes in both vaccinated and non-vaccinated clinical and subclinical cases mostly present stage IV encapsulated granulomas with central necrosis and mineralization.
6. PTB is endemic on the Canary Islands, with an average apparent prevalence of 18.4% with considerable variations between the affected farms. In farms with >20% initial seroprevalence, characteristics such as mean annual temperature, fencing and proximity to other farms could also play a role in antibody development.
7. The anti-MAP antibody development might be related to the age of the animals, although differences can exist between the age groups in herds with similar initial prevalences.

8. There are differences between the seroconversion after vaccination between young and adult animals. Although some young goats seem to not seroconvert as expected, in farms with >10% seroprevalence the immune response is heterogeneous among the age groups.
9. To design a protocol for early detection and assessment of natural caprine PTB cases in vaccinated and non-vaccinated animals, a combination of different tools including postmortem examination, laboratory tests for agent identification and serological control should be considered.

INTRODUCCIÓN

2. INTODUCCIÓN

2.1. LA GANADERÍA CAPRINA EN LA ACTUALIDAD

Para comprender la importancia de la ganadería caprina en la sociedad actual es imprescindible centrarse en el Objetivo de Desarrollo Sostenible 2 (ODS 2) de la Agenda 2030 de las Naciones Unidas que es "Hambre Cero" (Naciones Unidas, 2015). Además, el derecho a la alimentación junto con el derecho a la vida y la libertad, el trabajo y la educación están reconocidos por la Declaración Universal de Derechos Humanos y dos convenios internacionales jurídicamente vinculantes. A pesar del continuo esfuerzo para garantizarlos, la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO), en su último informe resalta que:

"Los agricultores del mundo producen alimentos suficientes para alimentar a más personas de la población mundial existente, sin embargo, el hambre persiste. Hasta 783 millones de personas se enfrentan al hambre debido a los conflictos, las repetidas crisis climáticas y las recesiones económicas. Esto repercute de manera más grave en los pobres y vulnerables, muchos de los cuales son hogares agrícolas, lo que refleja desigualdades cada vez mayores entre los países y dentro de ellos mismos." (FAO, 2023).

Según el último informe de la FAO, el hambre mundial ha sufrido un aumento repentino desde 2019, en el cual afectaba al 7,5%, a 2021 en el que alcanzó un 9,1%(1). Además, estos niveles aumentados se han mantenido durante 3 años consecutivos hasta el último informe de 2023, con tendencia de aumentar en África, manteniéndose estables en Asia y con progresos notables en América Latina. En porcentajes, en África se ve afectada un 20,4% de la población, frente a un 6,2% en América Latina y el Caribe, un 8,1% en Asia y un 7,3% en Oceanía. Sin

embargo, confirma el informe, más de la mitad de la población mundial que padece hambre sigue encontrándose en Asia(1) **(Figura 1)**.

Figura 1. Mapa del hambre. HugerMap LIVE. Programa Mundial de Alimentos (PMA)
Consultado: 19.08.2024

La oveja (*Ovis aries*) y la cabra (*Capra hircus*) fueron los primeros animales de producción domesticados alrededor de 10.000-8.000 a.C. en el suroeste asiático. Aunque comúnmente se les denomina "pequeños rumiantes", la cabra y la oveja difieren tanto a nivel genético, teniendo la cabra 60 cromosomas y la oveja 54, como a nivel etológico e incluso anatómico(2). Algunas características comportamentales caprinas se ven influenciadas por el tipo de producción a la que están sometidas, aunque hay aspectos como la estructura jerárquica del rebaño, el alto nivel de domesticación, su agilidad y habilidad para escalar y su ingenio para abrir cerraduras simples que son características de la especie(2).

Mahatma Gandhi, siglos después de ser domesticada, bautizó la cabra como "poor man´s cow" o "la vaca de los pobres". Este nombre se ha perpetuado hasta la actualidad ya que la producción caprina ha ayudado notablemente a combatir el hambre y abastecer de carne, leche y sus derivados, cuero y lana, a gran parte de los países con escasos recursos económicos y ha sido el pilar principal de su ganadería debido a su adaptabilidad a diversas condiciones, tanto climáticas, como territoriales. Actualmente, la cabra es el animal de producción con mayor distribución mundial. Según el informe de la FAO la población mundial de cabras domésticas (*Capra aegagrus hircus*) en 2022 era de aproximadamente 1,1 billones de animales, distribuidos en todos los continentes menos la Antártida, estando el 93,4% del censo centrado en Asia y África (**Figura 2**). El país con mayor población es la India con un total de 150 millones de cabras en 2022 (FAO, 2022).

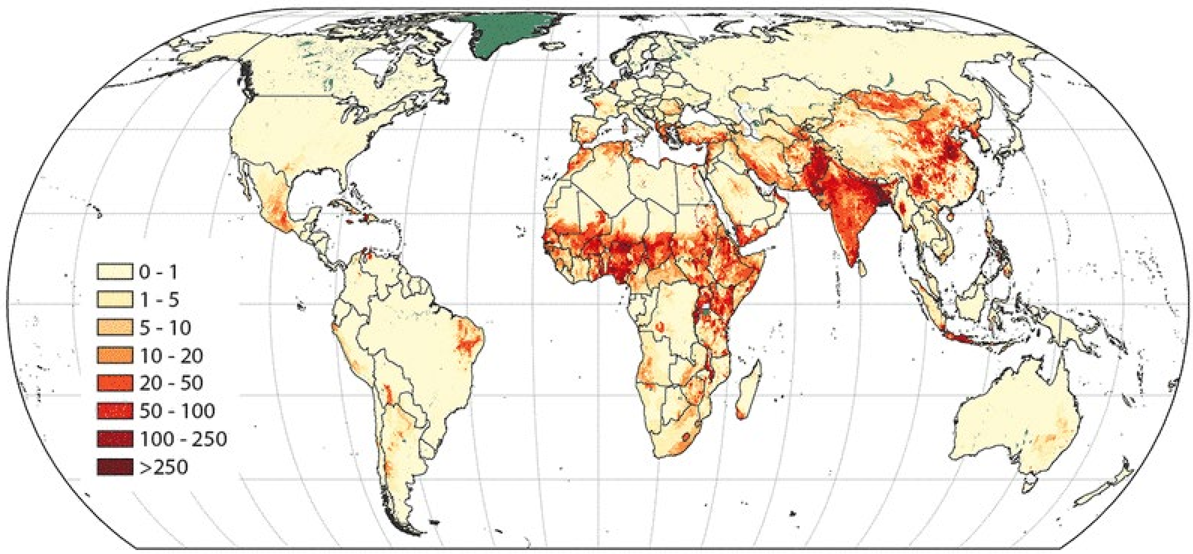


Figura 2. Número de cabras por m^2. FAO, 2015

Actualmente, Europa cuenta con la tercera población más numerosa de cabras del mundo (MAPA, 2023). España es el segundo mayor productor con un total de 2.293 millones de cabezas (MAPA, 2023) distribuidas mayoritariamente en las siguientes comunidades autónomas: Andalucía con 39%, Castilla-La Mancha con 16,7%, Extremadura con 10,7%, Canarias con 10.3% y Murcia con 9.7%(3) (**Figura 3**). En Canarias, el sector cuenta con un total de 200.054 cabezas distribuidas en 1256 explotaciones, con mayor censo en las islas de Fuerteventura y Gran Canaria (ISTAC 2023). El efectivo ganadero se compone principalmente de razas autóctonas certificadas que además están en peligro de extinción (Orden APM/26/2018). Actualmente, están reconocidas 3 razas de cabra canaria: Majorera, Tinerfeña y Palmera(4). La producción está dedicada principalmente a la leche para la elaboración de queso.

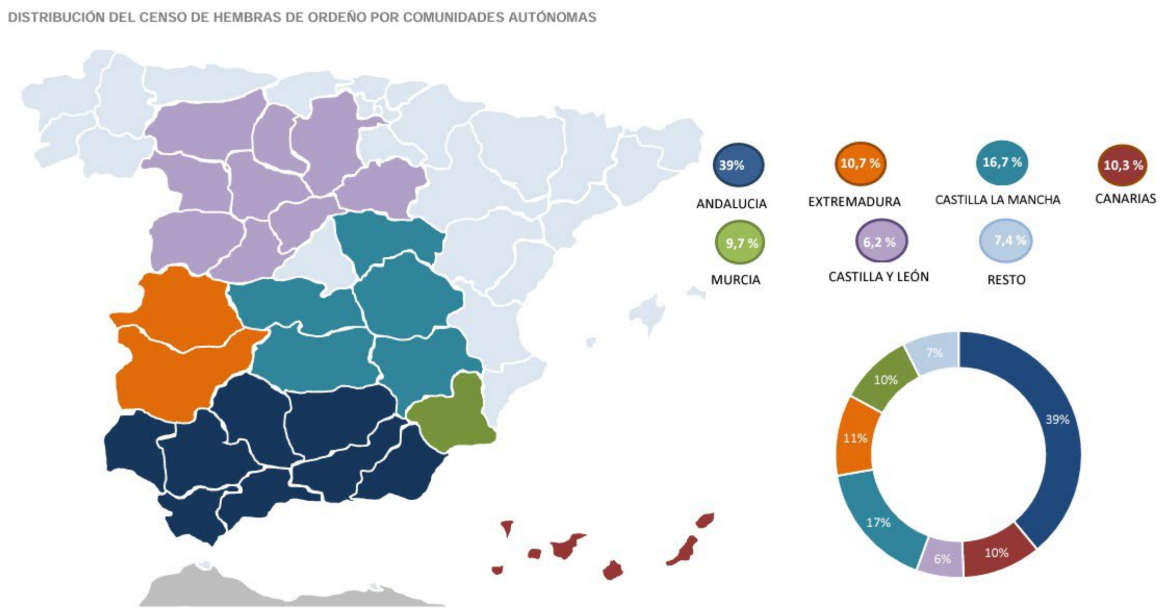


Figura 3. Distribución del censo de hembras caprinas de ordeño por comunidades autónomas en 2023. Ministerio de Agricultura, Pesca y Alimentación (MAPA)

El origen de las cabras canarias todavía no está confirmado, pero en la actualidad se ha demostrado, mediante análisis genéticos, que este es común entre las distintas razas isleñas(4,5). Se estima que su llegada se remonta al siglo I a.C. y coincide con el asentamiento en el archipiélago de los amazigh o bereberes que llegaron principalmente del Oeste y la parte central del Norte de África(6–8). Éstos trajeron consigo varios animales domesticados entre los cuales destacan las cabras, que eran una parte importante de su dieta. Posteriormente, dado que los aborígenes canarios no dominaban la navegación marítima, las islas y sus animales terrestres estuvieron aislados del continente africano hasta el siglo XV(4). Este hecho explica las diferencias en el haplotipo detectadas entre la población caprina canaria y la europea o la africana(5). Sin embargo, cabe destacar que a pesar de su origen común, las razas canarias se han adaptado exitosamente a los distintos microclimas existentes en las 8 islas y, por ejemplo, la raza Majorera y la Tinerfeña del sur está adaptadas al clima subtropical, y la Palmera y la Tinerfeña del norte a las condiciones de elevada humedad características de las isla de La Palma y del norte de la isla de Tenerife (4).

La producción caprina en el archipiélago está destinada principalmente a la producción de leche para la elaboración de quesos (9,10). Los quesos canarios forman parte del patrimonio de la Comunidad Autónoma, premiados en numerosas ocasiones a nivel nacional e internacional, convirtiéndose así en la “joya alimentaria de la gastronomía de Canarias”(9). En 2008, la calidad de los quesos canarios fue reconocida en el certamen de los “World Cheese Awards”. En el mismo año, el queso “Pimentón Curado de Arico” fue premiado como el “Mejor Queso Del Mundo” en la ceremonia celebrada en Dublín, República de Irlanda. El queso Majorero fue el primer queso de cabra español en conseguir la certificación de Denominación de Origen Protegida (DOP) en 1996(9,10). En el archipiélago se elaboran aproximadamente 17.000 toneladas de queso al año, principalmente de leche de cabra. Este producto se destina mayoritariamente al mercado interior (consumo medio anual de más de 9kg por habitante) aunque su importancia, debida a su calidad y singularidad, es cada vez mayor en el mercado nacional e internacional (9).

2.2. LA PARATUBERCULOSIS O LA ENFERMEDAD DE JOHNE

2.2.1. MARCO HISTÓRICO

Durante el siglo XIX se describieron por primera vez los signos clínicos y las lesiones anatomopatológicas asociados a la paratuberculosis (PTB) (11,12). El nombre de enfermedad de Johne se debe al trabajo publicado por H.A. Johne y L. Frothingham, que en 1895 demostraron la conexión entre la presencia de enteritis y de bacterias ácido-alcohol resistentes (BAAR) en tejido intestinal de una vaca siendo este el primer caso reportado de PTB (12). En 1906, Bang propuso el nombre "enteritis pseudotuberculosa" ya que logró distinguir entre enteritis tuberculosa y no tuberculosa (12). Sin embargo, el agente de la PTB, *Mycobacterium avium* subespecie *paratuberculosis* (MAP), se cultivó por primera vez en 1912 y, en 1914, se demostró que era capaz de producir enteritis en condiciones experimentales (12). Una vez que MAP se caracterizó por completo se incluyó en el género *Mycobacterium* con el nombre *Mycobacterium paratuberculosis* y consecutivamente la enfermedad fue nombrada "paratuberculosis". En 1911, dieciséis años después de la primera descripción en vacuno, se reportó por primera vez PTB en ovino por Stockman y, en cabras, en 1924 (11). A partir de las primeras descripciones, la PTB en pequeños rumiantes ha formado parte de la literatura veterinaria aunque siempre con menos frecuencia que la misma enfermedad en bovino (11).

2.2.2. AGENTE ETIOLÓGICO, GENOTIPOS Y SU PAPEL EPIDEMIOLÓGICO

El MAP es una bacteria bacilar, no móvil, de crecimiento lento, aerobia, Gram-positiva y ácido-alcohol resistente(13). Históricamente, las primeras descripciones de las distintas cepas se basaron en sus características epidemiológicas y fenotípicas y clasificaron el MAP en la cepa MAP-S (ovina, sheep strain) y la MAP-C (bovina, cattle strain) (13,14). Posteriormente, conforme avanzaron las técnicas de biología molecular, se identificaron dos tipos de MAP, tipo I y tipo II, ninguno exclusivo de la especie afectada(14,15). El MAP de tipo I es de crecimiento lento y afecta principalmente, pero no exclusivamente, al ganado ovino. El de tipo II es de crecimiento más rápido en comparación con el tipo I y principalmente afecta a bovino, aunque también se han descrito casos en caprino, ovino, ciervo y otros rumiantes (13,14). En cuanto al conocido como tipo III, en términos de crecimiento, es intermedio en comparación con los anteriores y se ha descrito en ovino, caprino, bovino y también en camélidos (13,14). Actualmente, mediante técnicas de secuenciación del genoma completo, este último tipo se ha reclasificado como subgrupo, junto con el tipo I, de la cepa MAP-S (14,15). Además, genotípicamente se describió, más recientemente, una tercera cepa de MAP, MAP-B (bisonte, bison) afectando a bisontes en Montana, EEUU (14). Sin embargo, una vez más, mediante secuenciación del genoma completo, esta última se reclasificó como subgrupo de la cepa MAP-C junto con el MAP aislado en bisontes en la India, llamado inicialmente "Indian Bison type" o "tipo B de la India" (14,15). En la **Figura 4** se esquematiza la clasificación actual basada en la secuenciación del genoma completo del MAP (16).

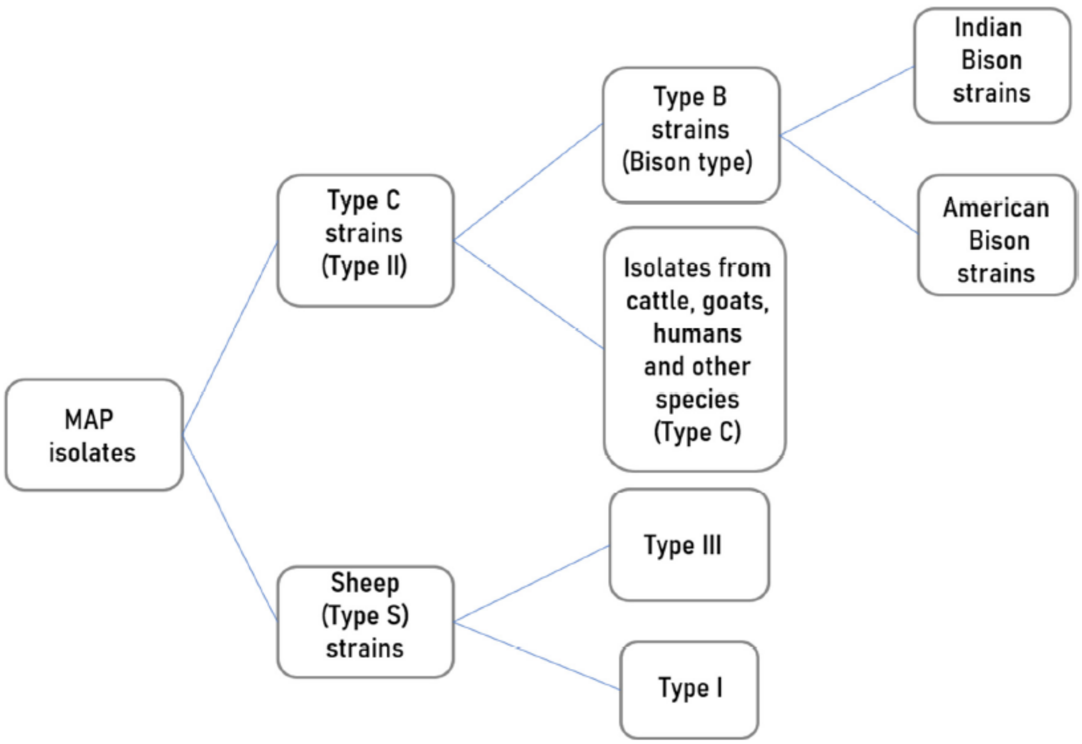


Figura 4. Esquema de la clasificación de MAP basada en análisis filogenético mediante secuenciación del genoma completo. Ssekitoleko y cols., 2021

Utilizando un amplio espectro de técnicas de identificación, se han detectado múltiples cepas dentro del mismo rebaño e incluso en un mismo animal(14). Por un lado, una de las aplicaciones más importantes de las técnicas de fenotipado es el rastreo de fuentes de infección. De esta manera, se ha podido aportar información valiosa para posteriormente instaurar medidas de control, manejo y prevención de la enfermedad a nivel de explotación (14). La explicación más extendida de la coexistencia de varias cepas se basa en la transmisión debida a la compra-venta de animales subclínicos infectados de distintos orígenes (14). Sin embargo, en un estudio, los autores proponen que infecciones con múltiples cepas de MAP pueden predisponer al desarrollo de enfermedad clínica (17). Además, otra posible explicación que se ha sugerido es la "microevolución" de la bacteria debido a su evolución crónica, tanto a nivel individuo, como a nivel de rebaño, lo cual podría conllevar a la gran diversidad genética descrita en la actualidad (18). Sin embargo, se necesitan futuros estudios para evaluar las posibles consecuencias de infecciones con múltiples cepas de MAP tanto a nivel individuo, como a nivel colectivo de rebaño (14).

2.2.3. PATOGENIA Y CLÍNICA

La PTB se ha descrito afectando mayoritariamente a rumiantes domésticos, aunque también a rumiantes salvajes e incluso a monogástricos (19–21). La patogenia consta de 3 fases: inicial, subclínica y clínica (19,21,22) (**Figura 5**).

La fase inicial es en la que se produce la infección. Los animales suelen infectarse en las primeras semanas de vida. Las principales vías de transmisión son la fecal-oral mediante consumo directo de MAP del suelo, agua o ubres contaminadas o la ingesta de calostro de madres infectadas, aunque también se ha descrito la infección *in utero*. Aunque la infección o reinfección en animales adultos ha sido reportada, esta es muy poco frecuente (19,21).

Una vez ingerida por el hospedador, MAP es absorbida en el intestino y transportada al tejido linfoide asociado a las mucosas dónde es fagocitada por macrófagos (12,21,23). Este mecanismo es facilitado por las células M del epitelio intestinal y por los enterocitos mediante un mecanismo dependiente de la fibrinonectina. Una vez activados, los macrófagos inician mecanismos microbicidas para combatir la entrada de MAP que incluyen el proceso de ingerir y la creación de fagosomas. Sin embargo, MAP, al igual que otras micobacterias, ha desarrollado varios mecanismos de supervivencia que le confieren su especial resistencia, entre los cuales destacan la inhibición de la maduración del fagosoma, interferencia con el mecanismo de apoptosis del macrófago y acidificación de fagosomas, además de su capacidad de evadir la presentación de antígenos por los macrófagos modulando la expresión de moléculas del Complejo Mayor de Histocompatibilidad (CMH) y regulando diferentes vías de señalización de la transducción. Debido a estas características, una vez MAP se instala dentro de las células del hospedador, empieza la fase subclínica que, por lo general, es larga y se caracteriza por la ausencia de signos clínicos (21).

La duración de la fase subclínica es variable y está caracterizada por una respuesta inmune celular con predominio de linfocitos Th1 (21). En ovino, los signos clínicos aparecen a partir de los 2 años de edad, aunque en algunos animales es a partir de los 4 años (24). En bovino la mayoría de casos clínicos se detectan entre los 2 y los 4 años de edad, aunque se han reportado casos de vacas asintomáticas infectadas hasta de 14 años de edad (24). En caprino, los datos sobre la duración de la fase subclínica son escasos aunque frecuentemente los signos clínicos aparecen antes que en bovino (25,26). Cabe destacar que los animales infectados que no presentan signos clínicos, pueden eliminar MAP al medio (19).

Una vez el animal pasa a la fase clínica, se produce el cambio de la población celular de linfocitos Th1 a Th2, la cual coincide con la aparición de signos clínicos que pueden variar entre especies (12,21,23). En bovino, la PTB se caracteriza por pérdida progresiva de peso y diarrea acuosa. En ovino y caprino, sin embargo, la diarrea no es un signo frecuente (2,19,20). Se estima que esta diferencia se debe a que las heces de los pequeños rumiantes son más secas que las de bovino y por tanto se necesitaría un disbalance más grave en la reabsorción de agua para que el animal llegue a tener diarrea (2). Sin embargo, se mantiene la pérdida de peso progresiva, que puede extenderse de semanas a meses terminando, en algunos casos, en emaciación (2,19) .

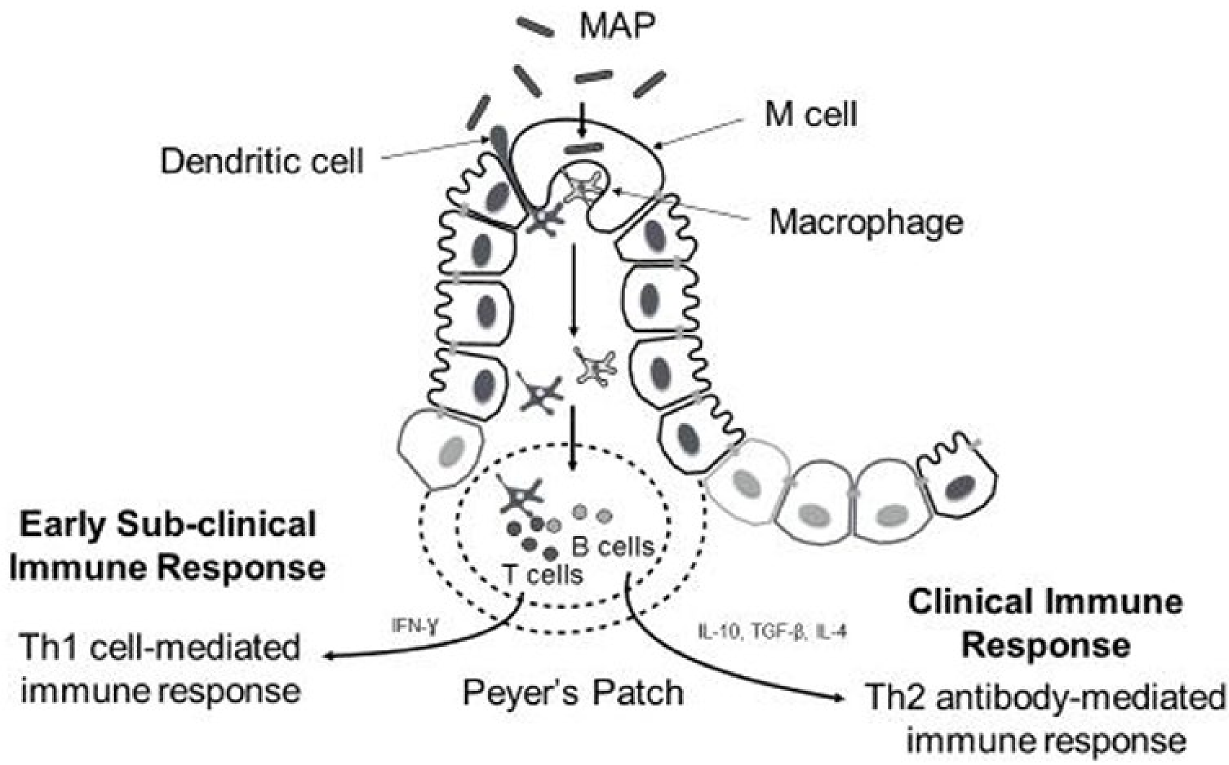


Figura 5. Representación de la absorción de MAP y la respuesta inmune durante la progresión de la enfermedad. Mallikarjunappa y cols, 2021.

2.2.4. DIAGNÓSTICO

El diagnóstico de la PTB, al igual que el de otras enfermedades causadas por micobacterias, es complicado y sigue siendo un reto científico(27). El principal impedimento es la larga fase subclínica que tiene la enfermedad (28). Sin embargo, aunque los test diagnósticos no tienen una alta sensibilidad, su uso puede ser beneficioso cuando se aplican acorde a la estrategia sanitaria para centrarse en la reducción de casos clínicos, la estimación de prevalencia, la reducción del impacto económico e incluso en la erradicación de la enfermedad en el rebaño(27). Una vez aclarado el objetivo, el protocolo se centraría en la identificación de animales que están en alguno de los siguientes estadios: "infectado" en el que MAP ha colonizado los tejidos del animal, "infeccioso" en el que el animal elimina MAP al medio o "afectado" que sería el caso de los animales que exhiben signos clínicos (27,28). Una vez aplicados los test diagnósticos, se pueden emplear más categorías para clasificar el caso de cada individuo, basados en los resultados, como se expone en la **Figura 6**.(22)

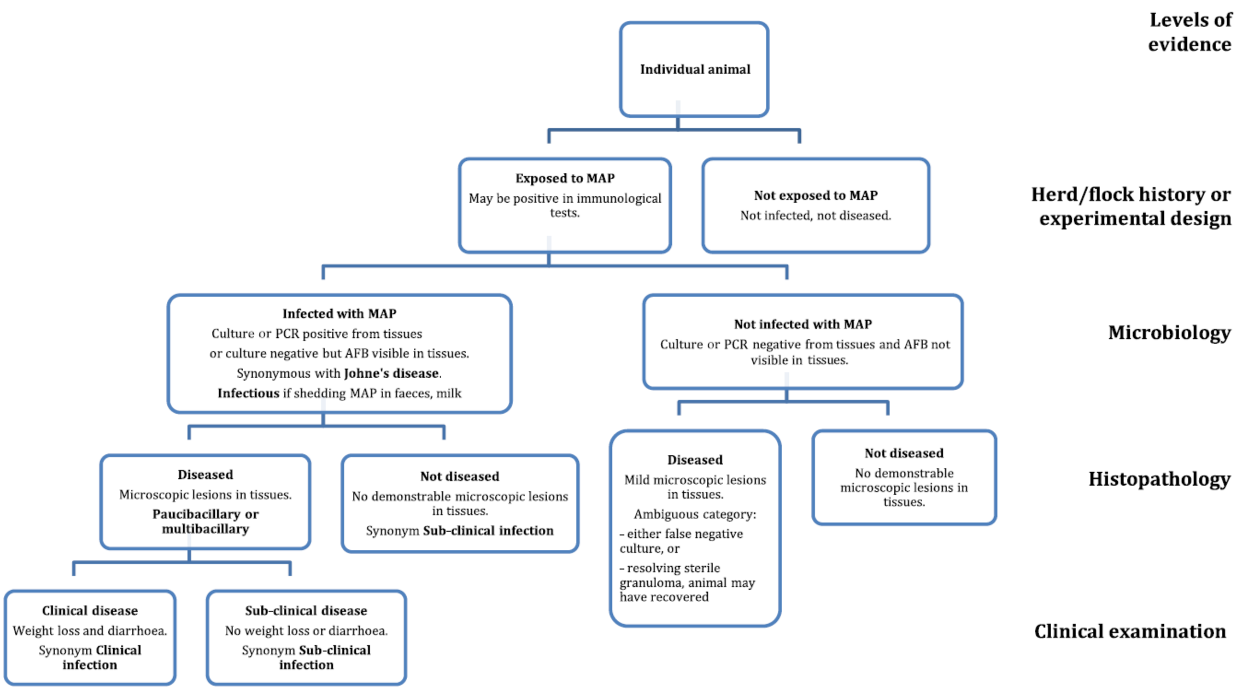


Figura 6. Clasificación dicotómica primaria de animales expuestos a MAP utilizando un enfoque diagnóstico sistemático y estructurado. AFB, bacilo ácido-alcohol resistente.
Whittington y cols., 2017.

Partiendo de la base de lo anteriormente expuesto, cabe destacar que existe una amplia gama de pruebas diagnósticas de PTB, cada una con sus limitaciones dependiendo de la fase de la enfermedad en la que se encuentra el animal(27) (**Figura 7**). Generalmente, los test se dividen en dos grandes grupos: directos e indirectos.

Table 1 Temporal applicability and accuracy of diagnostic tests for paratuberculosis in sheep and cattle

Test	Stage of disease when positive	Potential sensitivity[a]	Potential specificity[b]
Serum ELISA	Mid, late	Low to high[d]	Moderate to high
Delated type hypersensitivity	Early, mid	Moderate to high	Moderate
Interferon gamma assay	Early, mid	Unknown	Unknown
Faecal smear	Mid, late	Low[e]	Low to moderate
Faecal culture	Early[c], mid, late	Low to high[d]	High
Faecal qPCR	Early[c], mid, late	Moderate to high[f]	High
Tissue culture	Early, mid, late	High	High
Gross pathology	Late	Low to moderate	Low to moderate
Histopathology	Mid, late	Moderate to high	High
Clinical signs	Late	Low to moderate	Low to moderate

Adapted from Whittington and Sergeant (2001) [28] and Nielsen and Toft (2008) [30]. The terms low, moderate and high indicate ranges for sensitivity of <40%, 40–70% and >70%, respectively; corresponding values for specificity are <80%, 80–95% and >95%, respectively
[a]Proportion of truly infected/diseased animals that test positive
[b]Proportion of truly non-infected/healthy animals that test negative
[c]Transient, for a few months commencing a few months after infection
[d]High sensitivity possible in late stage of disease
[e]Due to low analytical sensitivity
[f]Based on sensitivity similar to culture in liquid medium

Figura 7. Aplicabilidad temporal y precisión de las pruebas de diagnóstico para la paratuberculosis en ovino y bovino. Whittington y cols, 2017

2.2.4.1. PRUEBAS DIAGNÓSTICAS DIRECTAS

Se clasifican como pruebas directas las que se centran en la identificación del agente causal, en este caso MAP. Entre ellas destacan: el cultivo bacteriano, la reacción en cadena de la polimerasa (PCR) y la identificación del agente en muestras tisulares mediante técnicas histoquímicas como el Ziehl-Neelsen o inmunohistoquímicas para identificación de antígenos de MAP.

2.2.4.1.1. CULTIVO BACTERIANO

El cultivo sigue considerándose el estándar de referencia (gold standard) para el diagnóstico de MAP, siendo las heces las muestras más habituales (19,24,27–29). Sin embargo, el cultivo de muestras tisulares ha demostrado ser la técnica con mayor sensibilidad para detectar muestras postivas(22). Cabe destacar que MAP es un agente difícilmente cultivable y de crecimiento lento que tarda entre 4 y 8 semanas hasta 16 semanas, dependiendo del medio de cultivo utilizado y la cepa, siendo la cepa S la de crecimiento más lento(22,30,31). Para el aislamiento de MAP se han utilizado clásicamente cultivos sólidos, siendo hoy en día el Herrold (Herrold's Egg Yolk Medium) y el Löwenstein- Jensen, los medios de elección(30,31). También se han descrito cultivos en medios con suero como el Dubos, pero estos, en la actualidad, están en desuso ya que los medios basados en yema de huevo fueron optimizados (30). Actualmente, también está ampliamente extendido el cultivo en medios líquidos, siendo la principal opción el Middlebrook 7H9, en el cual el periodo de incubación se reduce sin disminuir la sensibilidad. Por eso, estos últimos son la base para los protocolos automatizados de detección de MAP en laboratorios de diagnóstico veterinario (30).

2.2.4.1.2. PCR

Los diagnósticos de biología molecular, entre los que destaca la PCR, han sido los más recientemente desarrollados dentro del panel de diagnóstico habitual de la PTB, destacando por su especificidad y rapidez en muestras de heces y tejidos (19,22,24,32). El fragmento más comúnmente empleado para la amplificación es la secuencia de inserción 900 (IS 900) que está presente en gran número en el genoma del MAP (15–20 copias) (13,22,33–36). Sin embargo, se han identificado secuencias con muchas semejanzas en el ADN de otras micobacterias, lo cual ha conllevado al diseño de PCR para otros fragmentos más específicos como IS1311, ISMav2, ISMap02, f57 y hspX (30,31). No obstante, estos están presentes en el genoma de

MAP en menor número, lo cual podría reducir la sensibilidad de la PCR (30). Por tanto, hoy en día se tiende al uso de PCR para identificación de IS900 en combinación con otro fragmento para optimizar el diagnóstico (30). Es relevante destacar que, sobre todo en las muestras de heces, la detección de mayores cantidades de ADN de MAP indicaría la posibilidad de que el animal esté realmente infectado y no que se trate de MAP ingerido y excretado de manera pasiva (22).

2.2.4.1.3. EXAMEN ANATOMOPATOLÓGICO

El examen anatomopatológico es una herramienta fundamental para la confirmación del diagnóstico de PTB, tanto a nivel individuo, como a nivel de rebaño (11,19,20,37–39). Las lesiones características afectan a la mucosa intestinal, sobre todo a nivel de la válvula ileocecal (VIC) y a los linfonodos mesentéricos (LN MS) e ileocecal (LN IC), produciendo enteritis granulomatosa y linfoadenitis y linfangitis granulomatosas (20,37).

Los hallazgos macroscópicos, aunque no siempre presentes, son muy característicos y consisten en engrosamiento segmental o difuso de la mucosa, sobre todo de los tramos terminales de íleon y las primeras porciones del intestino grueso, aunque a veces se ven afectadas porciones del yeyuno e incluso del duodeno (11,19,20,38,40,41). El aspecto macroscópico que adquieren los tramos afectados es "cerebriforme" presentándose la mucosa marcadamente engrosada y corrugada. Aunque la VIC sea el primer sitio donde se pueden verificar lesiones histológicas, su afección macroscópica es variable. En cuanto a los linfonodos, estos se suelen presentar aumentados de tamaño de manera difusa (linfoadenomegalia), con coloración pálida y aspecto edematoso. Al corte, en algunos casos, se observa la formación de granulomas encapsulados con centros necróticos calcificados (11,19,20,38,40,41). Este tipo de lesiones es más común en el ganado caprino que en el bovino y se sugiere que son debidas a la alta sensibilidad de las cabras a infecciones micobacterianas, tanto la PTB como la tuberculosis (TB) causada por el complejo *Mycobacterium tuberculosis* (41). Es importante señalar que, en algunos casos, la linfangitis con trayectos linfáticos sinuosos y engrosados, visibles a nivel de los linfonodos, la serosa intestinal o el mesenterio son el único hallazgo macroscópico de PTB (20).

Dado que las lesiones macroscópicas no siempre están presentes y además no son exclusivas de la infección por MAP, el diagnóstico histopatológico es imprescindible para confirmar la PTB (20,41,42). A nivel del intestino y con especial énfasis en la VIC se observan lesiones caracterizadas por enteritis granulomatosa transmural con infiltrado de macrófagos espumosos con abundante citoplasma eosinófilo claro y número variable de células gigantes multinucleadas tipo Langhans, distribuidas de manera de focal a difusa, tanto en lámina propia, como en las placas de Peyer e incluso en la submucosa, la muscular y la serosa. A nivel de los linfonodos se observa linfoadenitis granulomatosa variando desde microgranulomas con focos de macrófagos espumosos hasta granulomas encapsulados con centros necróticos y calcificación extensa (11,19,20,38,40,41).

2.2.4.1.4. TINCIÓN DE ZIEHL-NEELSEN

Debido a las características de las bacterias del género *Mycobacterium* para su identificación, tanto en muestras tisulares como en improntas, se utilizan tinciones específicas para identificar bacterias ácido-alcohol resistentes. La técnica más utilizada en la tinción de Ziehl-Neelsen (ZN) mediante la cual el agente se tiñe de color magenta (19,21,22,43). Aunque esta técnica es ampliamente utilizada de manera rutinaria para la identificación de micobacterias, solamente identifica bacterias con pared intacta, lo cual explica su baja sensibilidad en casos en los que los bacilos que predominan en la muestra han perdido su pared celular. Además, el ZN confirma la presencia de micobacterias pero no identifica específicamente MAP (29,41,44–46).

2.2.4.1.5. INMUNOHISTOQUÍMICA

La eficacia en la identificación de antígenos de MAP mediante inmunohistoquímica (IHQ) utilizando anticuerpos policlonales ha sido confirmada por varios autores (29,45,46). Aunque algunos describen una concordancia buena entre la IHQ y el ZN, esta asociación se demuestra sobre todo en casos con elevado número de bacilos en las lesiones analizadas (29,41,44–46). Es importante puntualizar que la IHQ es capaz de detectar bacterias muertas con pared dañada, a diferencia del ZN. En algunos casos de PTB, como en otras micobacteriosis, se ha confirmado que, debido a la fuerte respuesta inmune humoral desencadenada, muchos de los bacilos presentes en las lesiones están muertos y han perdido su pared celular (29,41,44–46). Por tanto, la IHQ es una herramienta adicional confirmatoria de diagnóstico de PTB, aunque, teniendo en cuenta que los anticuerpos utilizados son policlonales, no se puede descartar la posibilidad de inmunomarcaje inespecífico (29,41,45,46).

2.2.4.2. PRUEBAS DIAGNÓSTICAS INDIRECTAS

La pruebas diagnósticas indirectas son las que se centran en la respuesta inmune del hospedador, la cual, en el caso de la PTB, es variable dependiendo del estadio de la enfermedad (22,27). La fase subclínica se caracterizan por estar mediada por una respuesta inmune celular (19,22). Para medirla en condiciones de campo se emplea comúnmente el test de intradermotuberculinización (IDTB) simple o comparada o el test *in vitro* para detección de interferón gamma (IFN-γ) (27,43). Posteriormente, en la fase clínica, se desencadena una respuesta inmune humoral para cuya detección, la prueba más ampliamente utilizada y de mayor sensibilidad es la enzimoinmunoanálisis de adsorción (ELISA, Enzyme-Linked Immunosorbent Assay), aunque se pueden utilizar también inmunodifusión en gel de agar (AGID,agar gel immunodiffusion) o el test de fijación del complemento (CFT, complement fixation test)(27,43). El CFT presenta valores altos de especificidad (90-99%), sin embargo, su sensibilidad es baja (18%-40%) (31). En cuanto al AGID, aunque durante muchos años fue ampliamente utilizada por su rapidez y bajo coste, su especificidad en caprino llega al 80% y su sensibilidad al 77,5% (31). Por estos factores, actualmente, ambas técnicas han sido reemplazadas por el ELISA (31) .

2.2.4.2.1. IDTB

La IDTB se lleva a cabo mediante la inoculación de proteína purificada derivada de *M. avium* (PPDa) o de Johnina (PPDj) y la posterior medición de la reacción de hipersensibilidad ocasionada en el punto de inoculación. También se han implementado IDTB comparadas inoculando al mismo animal PPDa y proteína purificada derivada de *Mycobacterium bovis* (PPDb) (43). Los animales con PTB presentan reacción en ambos puntos de inoculación, pero la del lado aviar es mayor. De esta manera, el diagnóstico de PTB en campo se puede llevar a cabo junto con los programas de erradicación de TB que incluyen la IDTB como prueba obligatoria (22,43). Las ventajas de estas técnicas consisten en la factibilidad de llevarlas a cabo en las granjas y la posibilidad de detección temprana. Sin embargo, como consecuencia de su baja sensibilidad (54% en bovino) y especificidad (79% en bovino, debida a la posible reacción cruzada), se recomienda que se usen solamente como prueba preliminar antes de iniciar el programa de control (43).

2.2.4.2.2. IFN-γ

Esta técnica consiste en la evaluación de producción de la citoquina IFN-γ por los linfocitos T después de una estimulación con PPD. La detección cuantitativa de IFN-γ puede utilizarse en bovinos entre 1 y 2 años de edad (43). La técnica presenta una sensibilidad del 80% en bovino y del 87% en ovino y caprino, sobre todo en fases iniciales de infección. Sin embargo, debido a su elevado coste y, sobre todo, al hecho de que las muestras se tienen que procesar a la mayor brevedad posible (antes de las 8 horas de recogida la muestra en bovinos) ya que las células tienen que estar vivas, no está ampliamente implementada en los programas de control de la PTB (43,47). Además, se han reportado reacciones cruzadas con otras micobacterias como las del complejo *Mycobacterium tuberculosis*, al igual que la IDTB (19,22,27).

2.2.4.2.3. ELISA

La detección de anticuerpos frente a MAP suele llevarse a cabo en muestras de suero y de leche(11,19,22,27,28). Existen varios kits en el mercado con sensibilidades entre 65% y 88% y especificidades alrededor del 99% (48). Aunque la serología tiene una sensibilidad baja, sobre todo en estadios iniciales de la enfermedad, en suero caprino, esta es relativamente superior a la reportada en ovino(13,48). Además, esta técnica está caracterizada por ser rápida, de relativamente bajo coste y es ampliamente utilizada sobre todo en campañas de control y vigilancia. (11,19,22,27,28) Los ELISA para leche, en su gran mayoría validados para bovino, han demostrado tener sensibilidades similares a los de suero aunque en caprino los datos son escasos (13,19,27).

2.2.5. CONTROL Y PREVENCIÓN

Para comprender la necesidad de control y prevención de la PTB, en primer lugar es imprescindible aclarar el impacto económico que tiene la enfermedad (11,27,31,49,50). Las pérdidas directas e indirectas han sido ampliamente estudiadas en bovino, tanto de leche como de carne, y, en menor medida, en pequeños rumiantes, sobre todo en oveja (27). En el ganado bovino se han descrito, entre otras, pérdidas indirectas por reducción de la producción de leche, reducción en la fertilidad y aumento en la susceptibilidad a otras enfermedades (27). En cuanto a las pérdidas directas, dada la pérdida de peso que sufren los animales afectados, se observa disminución en el peso de la canal. En términos económicos, en Estados Unidos se estima que cada vaca lechera infectada por PTB ocasiona pérdidas de entre 21US$ y 79US$, en Australia, 45A$ y 88$, en Canadá, 49CDN$, en Francia 234 € y en el Reino Unido, 27£[19]. En bovino de carne se estima que las pérdidas son menores. En pequeños rumiantes, la información es limitada aunque en Australia se ha reportado un promedio de entre 6,2% y 7,8% de mortalidad anual en ovino debido a la PTB, llegando en algunos casos al 20%(27). En el Reino Unido, se estiman unas pérdidas entre 0,4 y 32 millones de libras al año en la industria ovina por la PTB. En caprino los datos son más escasos, aunque en un estudio de Italia se reporta que la eficiencia de ganancias en ovino y caprino de leche baja de 85% a 64% por causa de infección con MAP(51). Por último, también resaltar las posibles pérdidas económicas que tendría la implementación de restricciones de mercado en el caso de regiones con campañas de control y vigilancia(27).

Basado en lo anteriormente expuesto y la distribución mundial de la enfermedad, se han desarrollado varios programas de control que incluyen una o más de las estrategias que se resumen a continuación (19):

2.2.5.1. DIAGNÓSTICO Y SACRIFICO

Esta estrategia está relacionada con la aplicación de pruebas diagnósticas efectivas aplicadas en repetidas ocasiones con el fin de identificar animales en estadios iniciales de la infección (11,13,19,27). En el caso de la PTB, las herramientas disponibles tienen una sensibilidad baja, sobre todo en los estadios tempranos y subclínicos de la enfermedad (19). A pesar de este dato, sorprendentemente esta estrategia de control ha sido ampliamente utilizada, sobre todo en bovino, aunque conlleva un elevado coste (19,27). Sin embargo, en ovino y caprino, aplicar solamente la estrategia de diagnóstico y sacrificio resulta inviable económicamente ya que el precio del animal no compensa el elevado coste de los test (11,19). No obstante, combinada con otras estrategias, como el cambio en las medidas de bioseguridad, podría ser una herramienta valiosa de control de la infección y diseminación de MAP (19).

2.2.5.2. AUMENTO DE LAS MEDIDAS DE BIOSEGURIDAD

Las adaptaciones introducidas en las medidas de bioseguridad en la granja para la prevención y control de PTB se basan en la separación de las crías de las madres, control del calostro y de la leche para asegurar que están libres de MAP, limpieza exhaustiva del entorno, cría y recría adecuadas, cría de animales de reposición en ambiente libre de MAP junto con un aumento generalizado de la higiene de la exploración (19). Para la correcta implementación de estas prácticas, sin embargo, hacen falta conocimientos avanzados, diligencia y una considerable inversión económica que no siempre es factible en los rebaños de ovino y caprino, sobre todo cuando el objetivo es conseguir resultados inmediatos o a corto plazo (19,52). También cabe destacar que, en ganadería extensiva, uno de los principales factores a tener en cuenta en ovino es la vigilancia de los animales que pierden peso, para eliminar la posible contaminación del medio por la alta tasa de excreción de MAP por parte de animales con clínica de PTB. En España, estudios recientes en ganado caprino, resaltan como factores de riesgo asociados a la seroprevalencia los siguientes: producción intensiva, ausencia de separación y manejo por lotes, ventilación inapropiada, positividad a pruebas para detectar el virus de la artritis-encefalitis caprina (CAEV) y la ausencia de vallado (53,54).

2.2.5.3. VACUNACIÓN FRENTE A MAP

La primera vacuna contra PTB fue viva-atenuada, siendo ampliamente utilizada hasta los años 60, cuando se demostró que la eficacia de las vacunas inactivadas era igual y, por razones de bioseguridad, estas últimas reemplazaron a las vivas-atenuadas(11). El mecanismo de protección que confiere la vacuna no está bien dilucidado (11). Además, se ha demostrado, tanto en bovino, como en ovino y caprino, que la vacunación no previene la infección pero si reduce la excreción de MAP al medio y conlleva a una disminución significativa de la severidad de las lesiones observadas (11,19,27,42,55).

Actualmente, la vacunación está sujeta a permisos especiales en algunos países como Francia, Alemania y España, y prohibida en otros como Dinamarca, ya que el control de la TB es de importancia internacional y se ha demostrado que la vacunación frente a MAP interfiere con las pruebas de diagnóstico oficiales de TB como la IDTB (11,19,27). Sin embargo, históricamente, la implementación de la vacunación fue un éxito y en ovino es prácticamente la única estrategia de control ampliamente instaurada, descartando el caso del intento de erradicación de la enfermedad en Australia en los años 90, que no tuvo resultados satisfactorios y conllevó a considerables pérdidas económicas (11). Inicialmente, en España se consideraba que el caprino, al igual que el ovino, era relativamente resistente a la PTB y la vacunación fue ampliamente utilizada en ambas especies(11). Sin embargo se demostró que, las cabras, con diferencia al ganado ovino, son muy sensibles tanto a la TB como a la PTB y la vacunación sin restricciones en muchas regiones conllevó a confusiones y a la imposibilidad de evaluar correctamente tanto el efecto de la vacunación como el control de la TB (11). Actualmente, en algunas zonas del país, como en las Islas Canarias, donde se centra la presente tesis doctoral, la vacunación frente a PTB está sujeta a legislación específica local que requiere de los ganaderos certificar la ausencia de TB y la presencia de PTB en la granja para obtener el permiso de vacunación (Decreto 51/2018 del 23 de abril). Estas restricciones vienen a raíz del estatus de "oficialmente indemne" de TB que tiene el archipiélago desde el año 2017.

Actualmente, la PTB está en la lista de enfermedades de declaración obligatoria de la Organización Mundial de la Sanidad Animal (OMSA) y debe ser notificada según las instrucciones del Código Sanitario para los Animales Terrestres. Además, a nivel de la Unión Europea (UE), la PTB está en la categoría E, enfermedades sobre las que es necesario que la Unión ejerza vigilancia (Regulation (EU) 2018/1882), como se detalla en la regulación sobre enfermedades transmisibles de los animales y por la que se modifican o derogan algunos actos en materia de sanidad animal (Artículo 9(1)(e) de la Regulation (EU) 2016/42). Sin embargo, tanto a nivel europeo como a nivel nacional, la PTB se considera subnotificada. Además, varios trabajos recientes resaltan la escasa información científica sobre la PTB en caprino y más a nivel de España.

Considerando esto último, junto con todo lo anteriormente expuesto, en la presente tesis doctoral se establecen los objetivos.

3. BIBLIOGRAFÍA

1. FAO, FIDA, UNICEF, PMA y OMS. *Versión resumida de El estado de la seguridad alimentaria y la nutrición en el mundo 2024: Financiación para poner fin al hambre, la inseguridad alimentaria y todas las formas de malnutrición*. Roma (2024). doi: 10.4060/cd1276es

2. Smith MC, Sherman DM. *Goat medicine*. John Wiley & Sons. (2009).

3. Resultados de las Encuestas de Ganado Ovino-Caprino” Edita: © Ministerio de Agricultura, Pesca y Alimentación. NIPO: 00322136X Catálogo de Publicaciones de la Administración General del Estado: https://cpage.mpr.gob.es/

4. Senczuk G, Macrì M, Di Civita M, Mastrangelo S, del Rosario Fresno M, Capote J, Pilla F, Delgado JV, Amills M, Martínez A. The demographic history and adaptation of Canarian goat breeds to environmental conditions through the use of genome-wide SNP data. *Genetics Selection Evolution* (2024) 56: doi: 10.1186/s12711-023-00869-0

5. Ferrando A, Manunza A, Jordana J, Capote J, Pons A, Pais J, Delgado T, Atoche P, Cabrera B, Martínez A, y cols. A mitochondrial analysis reveals distinct founder effect signatures in Canarian and Balearic goats. *Anim Genet* (2015) 46:452–456. doi: 10.1111/age.12302

6. Rodríguez-Varela R, Günther T, Krzewińska M, Storå J, Gillingwater TH, MacCallum M, Arsuaga JL, Dobney K, Valdiosera C, Jakobsson M, y cols. Genomic analyses of pre-European conquest human remains from the Canary Islands reveal close affinity to modern North Africans. *Current Biology* (2017) 27:3396-3402.e5. doi: 10.1016/j.cub.2017.09.059

7. Fregel R, Ordóñez AC, Santana-Cabrera J, Cabrera VM, Velasco-Vázquez J, Alberto V, Moreno-Benítez MA, Delgado-Darias T, Rodríguez-Rodríguez A, Hernández JC, y cols. Mitogenomes illuminate the origin and migration patterns of the indigenous people of the Canary Islands. *PLoS One* (2019) 14: doi: 10.1371/journal.pone.0209125

8. Maca-Meyer N, Arnay M, Rando JC, Flores C, González AM, Cabrera VM, Larruga JM. Ancient mtDNA analysis and the origin of the Guanches. *European Journal of Human Genetics* (2004) 12:155–162. doi: 10.1038/sj.ejhg.5201075

9. Guía de quesos de Canarias. Edita: Instituto Canario de Calidad Agroalimentaria del Gobierno de Canarias.(2012)

10. Fresno M, Álvarez S. Chemical, textural and sensorial changes during the ripening of Majorero goat cheese. *Int J Dairy Technol* (2012) 65:393–400. doi: 10.1111/j.1471-0307.2012.00842.x

11. Juste RA, Perez V. Control of paratuberculosis in sheep and goats. *Veterinary Clinics of North America - Food Animal Practice* (2011) 27:127–138. doi: 10.1016/j.cvfa.2010.10.020

12. Cocito C, Gilot P, Coene M, De Kesel M, Poupart P, Vannuffel P. Paratuberculosis. (1994). 328–345 p.

13. Idris SM, Eltom KH, Okuni JB, Ojok L, Elmagzoub WA, Wahed AA El, Eltayeb E, Gameel AA. Paratuberculosis: The hidden killer of small ruminants. *Animals* (2022) 12: doi: 10.3390/ani12010012

14. Fawzy A, Zschöck M, Ewers C, Eisenberg T. Genotyping methods and molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). *Int J Vet Sci Med* (2018) 6:258–264. doi: 10.1016/j.ijvsm.2018.08.001

15. Bryant JM, Thibault VC, Smith DGE, McLuckie J, Heron I, Sevilla IA, Biet F, Harris SR, Maskell DJ, Bentley SD, y cols. Phylogenomic exploration of the relationships between strains of *Mycobacterium avium* subspecies *paratuberculosis*. *BMC Genomics* (2016) 17: doi: 10.1186/s12864-015-2234-5

16. Ssekitoleko J, Ojok L, Wahed AA El, Erume J, Amanzada A, Eltayeb E, Eltom KH, Okuni JB. *Mycobacterium avium* subsp. *paratuberculosis* virulence: a review. *Microorganisms* (2021) 9: doi: 10.3390/microorganisms9122623

17. Gerritsmann H, Stalder GL, Spergser J, Hoelzl F, Deutz A, Kuebber-Heiss A, Walzer C, Smith S. Multiple strain infections and high genotypic diversity among *Mycobacterium avium* subsp. *paratuberculosis* field isolates from diseased wild and domestic ruminant species in the eastern Alpine region of Austria. *Infection, Genetics and Evolution* (2014) 21:244–251. doi: 10.1016/J.MEEGID.2013.11.009

18. Van Hulzen KJE, Heuven HCM, Nielen M, Hoeboer J, Santema WJ, Koets AP. Different *Mycobacterium avium* subsp. *paratuberculosis* MIRU–VNTR patterns coexist within cattle herds. *Vet Microbiol* (2011) 148:419–424. doi: 10.1016/J.VETMIC.2010.09.029

19. Windsor PA. Paratuberculosis in sheep and goats. *Vet Microbiol* (2015) 181:161–169. doi: 10.1016/j.vetmic.2015.07.019

20. Uzal FA, Plattner BL, Hostetter JM. "Chapter 1 - Alimentary System.," En: Maxie MG, editor. *Jubb, Kennedy & Palmer's Pathology of Domestic Animals: Volume 2 (Sixth Edition)*. W.B. Saunders (2016). p. 1-257.e2 doi: https://doi.org/10.1016/B978-1-7020-5318-4.00007-3

21. Mallikarjunappa S, Brito LF, Pant SD, Schenkel FS, Meade KG, Karrow NA. Johne's disease in dairy cattle: an immunogenetic perspective. *Front Vet Sci* (2021) 8: doi: 10.3389/fvets.2021.718987

22. Whittington RJ, Begg DJ, de Silva K, Purdie AC, Dhand NK, Plain KM. Case definition terminology for paratuberculosis (Johne's disease). *BMC Vet Res* (2017) 13: doi: 10.1186/s12917-017-1254-6

23. Sweeney RW. Pathogenesis of paratuberculosis. *Veterinary Clinics of North America - Food Animal Practice* (2011) 27:537–546. doi: 10.1016/j.cvfa.2011.07.001

24. Whittington RJ, Sergeant E. Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp *paratuberculosis* in animal populations. *Aust Vet J* (2001) 79:267–278. doi: 10.1111/j.1751-0813.2001.tb11980.x

25. Stewart DJ, Vaughan JA, Stiles PL, Noske PJ, Tizard MLV, Prowse SJ, Michalski WP, Butler KL, Jones SL. A long-term bacteriological and immunological study in Holstein-Friesian cattle experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis* and necropsy culture results for Holstein-Friesian cattle, Merino sheep and Angora goats. *Vet Microbiol* (2007) 122:83–96. doi: 10.1016/j.vetmic.2006.12.030

26. Krüger C, Köhler H, Liebler-Tenorio EM. Sequential development of lesions 3, 6, 9, and 12 months after experimental infection of goat kids with *Mycobacterium avium* subsp *paratuberculosis*. *Vet Pathol* (2015) 52:276–290. doi: 10.1177/0300985814533804

27. Whittington R, Donat K, Weber MF, Kelton D, Nielsen SS, Eisenberg S, Arrigoni N, Juste R, Sáez JL, Dhand N, y cols. Control of paratuberculosis: Who, why and how. A review of 48 countries. *BMC Vet Res* (2019) 15: doi: 10.1186/s12917-019-1943-4

28. Nielsen SS, Toft N. Ante mortem diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon-γ assay and faecal culture techniques. *Vet Microbiol* (2008) 129:217–235. doi: 10.1016/j.vetmic.2007.12.011

29. Martinson SA, Hanna PE, Ikede BO, y cols. Comparison of bacterial culture, histopathology, and immunohistochemistry for the diagnosis of Johne's disease in culled dairy cows. *J Vet Diagn Invest*. 2008;20(1):51-57. doi:10.1177/104063870802000109

30. Dane H, Stewart LD, Grant IR. Culture of *Mycobacterium avium* subsp. *paratuberculosis*: challenges, limitations and future prospects. *J Appl Microbiol* (2023) 134: doi: 10.1093/jambio/lxac017

31. De Juan Ferré L , Domínguez Rodríguez L, Suárez Fernández G, Mateos García A. *Paratuberculosis caprina : aportaciones a su diagnóstico, epidemilogía molecular y control*. Madrid: Universidad Complutense de Madrid. (2005). 291 p.

32. Pillai SR, Jayarao BM. Application of IS900 PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* directly from raw milk. *J Dairy Sci* (2002) 85:1052–1057. doi: 10.3168/jds.S0022-0302(02)74165-9

33. Espinosa J, Fernández M, Royo M, Grau A, Ángel Collazos J, Benavides J, del Carmen Ferreras M, Mínguez O, Pérez V. Influence of vaccination against paratuberculosis on the diagnosis of caprine tuberculosis during official eradication programmes in Castilla y León (Spain). *Transbound Emerg Dis* (2021) 68:692–703. doi: 10.1111/tbed.13732

34. Gandhi H, Jain M, Gupta S, Singh AK, Kumar A, Sohal JS. Comparative evaluation of various in-house protocols on diagnostic performance for paratuberculosis IS900 PCR. *Mol Biol Rep* (2023) 50:943–947. doi: 10.1007/s11033-022-07984-z

35. Conde C, Price-Carter M, Cochard T, Branger M, Stevenson K, Whittington R, Bannantine JP, Biet F. Whole-genome analysis of *Mycobacterium avium* subsp. *paratuberculosis* IS900 Insertions Reveals Strain Type-Specific Modalities. *Front Microbiol* (2021) 12: doi: 10.3389/fmicb.2021.660002

36. Slana I, Kralik P, Kralova A, Pavlik I. On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. *Int J Food Microbiol* (2008) 128:250–257. doi: 10.1016/j.ijfoodmicro.2008.08.013

37. Gelberg HB. "Chapter 7 - Alimentary System and the Peritoneum, Omentum, Mesentery, and Peritoneal Cavity1.," En: Zachary JF, editor. *Pathologic Basis of Veterinary Disease (Sixth Edition)*. Mosby (2017). p. 324-411.e1 doi: https://doi.org/10.1016/B978-0-323-35775-3.00007-2

38. Derakhshandeh A, Namazi F, Khatamsaz E, Eraghi V, Hemati Z. Goat paratuberculosis in Shiraz: Histopathological and molecular approaches. *Veterinary Research Forum* (2018) 9:253–257. doi: 10.30466/vrf.2018.32080

39. Benavides J, González L, Dagleish M, Pérez V. Diagnostic pathology in microbial diseases of sheep or goats. *Vet Microbiol* (2015) 181:15–26. doi: 10.1016/j.vetmic.2015.07.012

40. Khodakaram Tafti A, Rashidi K. The pathology of goat paratuberculosis: Gross and histopathological lesions in the intestines and mesenteric lymph nodes. *Journal of Veterinary Medicine, Series B* (2000) 47:487–495. doi: 10.1046/j.1439-0450.2000.00375.x

41. Corpa JM, Garrido J, García Marín JF, Pérez V. Classification of lesions observed in natural cases of paratuberculosis in goats. *J Comp Pathol* (2000) 122:255–265. doi: 10.1053/jcpa.1999.0368

42. Corpa JM, Pérez V, García Marín JF, Sánchez MA. Control of paratuberculosis (Johne's disease) in goats by vaccination of adult animals. *Veterinary Record* (2000) 146:195–196. doi: 10.1136/vr.146.7.195

43. Gilardoni LR, Paolicchi FA, Mundo SL. Bovine paratuberculosis: a review of the advantages and disadvantages of different diagnostic tests. *Rev Argent Microbiol* (2012) 44:201–215.

44. Delgado L, Marín JFG, Muñoz M, Benavides J, Juste RA, García-Pariente C, Fuertes M, González J, Ferreras MC, Pérez V. Pathological findings in young and adult sheep following experimental infection with 2 different doses of *Mycobacterium avium* subspecies *paratuberculosis*. *Vet Pathol* (2013) 50:857–866. doi: 10.1177/0300985813476066

45. Thoresen OF, Falk K, Evensen Ø. Comparison of immunohistochemistry, acid-fast staining, and cultivation for detection of Mycobacterium paratuberculosis in goats. (1994). 195–199 p.

46. Kheirandish R, Tafti AK, Hosseini A. Classification of lesions and comparison of immunohistochemical and acid-fast staining in diagnosis of naturally occurring paratuberculosis in goats. *Small Ruminant Research* (2009) 87:81–85. doi: 10.1016/j.smallrumres.2009.09.030

47. Bezos J, Romero B, de Juan L, González S, Sáez JL. Manual de procedimiento para la realización de las pruebas de intradermotuberculinización e interferón-gamma. Edita:®Centro de Vigilancia Sanitaria Veterinaria (VISAVET). UCM. y la Subdirección General de Sanidad e Higiene Animal y Trazabilidad. MAPA. (2023).

48. Whittington RJ, Eamens GJ, Cousins D V. Specificity of absorbed ELISA and agar gel immunodiffusion tests for paratuberculosis in goats with observations about use of these tests in infected goats. *Aust Vet J* (2003) 81:71–75. doi: 10.1111/j.1751-0813.2003.tb11437.x

49. Bastida F, Juste RA. Paratuberculosis control: A review with a focus on vaccination. *J Immune Based Ther Vaccines* (2011) 9: doi: 10.1186/1476-8518-9-8

50. Garcia A, Shalloo L. Invited review: The economic impact and control of paratuberculosis in cattle. *J Dairy Sci* (2015) 98:5019–5039. doi: 10.3168/jds.2014-9241

51. Sardaro R, Pieragostini E, Rubino G, Petazzi F. Impact of *Mycobacterium avium* subspecies *paratuberculosis* on profit efficiency in semi-extensive dairy sheep and goat farms of Apulia, southern Italy. *Prev Vet Med* (2017) 136:56–64. doi: 10.1016/j.prevetmed.2016.11.013

52. Djønne B. "Paratuberculosis in goats" En: *Paratuberculosis: organism, disease, control*. Wallingford: CABI (2010). p. 169–178

53. Barrero-Domínguez B, Luque I, Huerta B, Gomez-Laguna J, Galán-Relaño Á, Gómez-Gascón L, Sánchez M, Astorga RJ. Paratuberculosis in dairy goat flocks from southern Spain: risk factors associated with seroprevalence. *Veterinary Record* (2019) 185: doi: 10.1136/vetrec-2019-105465

54. Jiménez-Martín D, García-Bocanegra I, Risalde MA, Fernández-Molera V, Jiménez-Ruiz S, Isla J, Cano-Terriza D. Epidemiology of paratuberculosis in sheep and goats in southern Spain. *Prev Vet Med* (2022) 202: doi: 10.1016/j.prevetmed.2022.105637

55. Corpa JM, Pérez V, García Marín JF. Differences in the immune responses in lambs and kids vaccinated against paratuberculosis, according to the age of vaccination. *Vet Microbiol*. 2000;77(3-4):475-485. doi:10.1016/s0378-1135(00)00332-1

OBJETIVOS

4. OBJETIVOS/OBJECTIVES

1. Evaluar las lesiones morfológicas asociadas con la PTB clínica y subclínica en rebaños caprinos en infección natural.

   Evaluate the morphological lesions associated with clinical and subclinical paratuberculosis in naturally infected goat herds.

2. Describir los diferentes hallazgos patológicos concomitantes en cabras naturalmente infectadas con paratuberculosis (PTB).

   Recognize the different concomitant pathological findings in goats naturally infected with PTB.

3. Implementar un diagnóstico más efectivo para identificar MAP utilizando técnicas de inmunohistoquímica y biología molecular.

   Implement a more effective diagnosis for identifying MAP using immunohistochemical and molecular biology techniques.

4. Estudiar el impacto de la vacunación anti-MAP en la identificación etiológica, la gravedad y distribución de las lesiones en casos clínicos y subclínicos de infección natural de PTB caprina.

   Study the impact of anti-MAP vaccination on etiological identification, lesion severity and distribution in clinical and subclinical cases of natural infection with caprine PTB.

5. Determinar la prevalencia de la paratuberculosis en rebaños caprinos de las Islas Canarias, España, aplicando pruebas serológicas. Evaluar la respuesta inmunitaria en relación con la edad, el entorno y las características de la explotación.

   Determine the prevalence of paratuberculosis in goat herds across the Canary Islands, Spain applying serological assay. Evaluate the immune response in relation to age, environment and farm characteristics.

6. Analizar las respuestas inmunitarias a la vacunación anti-MAP en relación con la edad en rebaños naturalmente infectados con PTB en las Islas Canarias, España.

   Analyze immune responses to anti-MAP vaccination in relation to age in PTB naturally infected herds on the Canary Islands, Spain.

CAPÍTULO I

Evaluación morfológica de lesiones concomitantes detectadas en rebaños caprinos con infección natural de pararatuberculosis (enfermedad de Johne)

5. CAPÍTULO I

5.1. RESUMEN

Evaluación morfológica de lesiones concomitantes detectadas en rebaños caprinos con infección natural de pararatuberculosis (enfermedad de Johne)

Morphological assessment of concomitant lesions detected in goat herds naturally infected with paratuberculosis (Johne's disease)

La paratuberculosis (PTB) o enfermedad de Johne es una enfermedad crónica causada por *Mycobacterium avium* subespecie *paratuberculosis* (MAP), que provoca pérdidas económicas significativas en la industria ganadera. El estudio aborda la detección de PTB caprina en rebaños vacunados y no vacunados, con un enfoque en el diagnóstico morfológico, la clasificación de lesiones y la identificación bacteriana. Se analizaron 39 cabras adultas infectadas de forma natural, de las cuales 15 estaban vacunadas y 24 no vacunadas. La investigación se centró en identificar las lesiones concomitantes y las lesiones inducidas por PTB en los órganos diana, como los linfonodos y el sistema gastrointestinal, evaluando el impacto de la vacunación en el tipo y gravedad de lesiones producidas.

Los animales se seleccionaron de 8 granjas de las Islas Canarias con historial de PTB. Se realizó necropsia y examen histopatológico para evaluar las lesiones en linfonodos mesentéricos (LN MS), válvula ileocecal (VIC), y otros órganos. Las muestras se fijaron en formalina y se tiñeron con hematoxilina/eosina (HE) y Ziehl-Neelsen (ZN) para la detección de bacilos ácido-alcohol resistentes (BAAR). Además, se empleó inmunohistoquímica (IHQ) con anticuerpo policlonal para identificar antígenos de MAP en las muestras tisulares. Todos los animales incluidos presentaban lesiones histopatológicas de enteritis granulomatosa a nivel de la VIC y/o linfoadenitis granulomatosa en los LN MS.

Macroscópicamente, el 61,5% de las cabras presentaron lesiones granulomatosas en LN MS y el 17,9% en la VIC. Las lesiones macroscópicas fueron más prevalentes en los animales no vacunados. Además, los animales no vacunados exhibieron enteritis granulomatosa variando de leve a marcada, en comparación con los vacunados, en los que solamente se observaron lesiones leves. Los granulomas a nivel de los LN MS fueron predominantemente de grado IV tanto en los vacunados, como en los no vacunados.

Las patologías concomitantes detectadas fueron mayoritariamente de origen inflamatorio, afectando al sistema respiratorio, gastrointestinal y hemolinfático. Se observó una mayor prevalencia de procesos inflamatorios a nivel pulmonar en los no vacunados (70,8%), en comparación con los vacunados (53,3%). Además, existió diferencia significativa en el grupo de los no vacunados, en el cual, mayor número de animales con enteritis granulomatosa presentaba también lesiones histológicas de neumonía ($p=0,027$). La inflamación pulmonar fue confirmada en animales no vacunados de todos los rangos de edad, en comparación con los vacunados, entre los cuales solamente se detectó en los de (12-24] meses de edad ($p=0,035$).

El estudio concluye que la PTB puede estar asociada con diversas patologías concomitantes, principalmente inflamatorias, la mayoría detectables únicamente después de un examen histopatológico. Estos resultados destacan la importancia del examen histológico rutinario, así como el valor de la anatomía patológica como una herramienta útil para el diagnóstico y manejo de la PTB en rebaños de cabras.

La vacunación contra MAP parece tener un efecto beneficioso en la reducción de estas patologías, basándonos en los hallazgos descritos y en las diferencias estadísticamente significativas demostradas entre los animales vacunados y no vacunados. Aunque la vacunación no elimina completamente las infecciones por MAP, puede reducir la gravedad de las lesiones inducidas por la bacteria, lo que podría tener un impacto positivo en la salud general del rebaño y las pérdidas económicas.

animals

IMPACT FACTOR 2.7
Indexed in: PubMed
CITESCORE 4.9

Article

Morphological Assessment of Concomitant Lesions Detected in Goat Herds Naturally Infected with Paratuberculosis (Johne's Disease)

Elena Plamenova Stefanova, Óscar Quesada-Canales, Yania Paz-Sánchez, María José Caballero, María del Pino Quintana-Montesdeoca, Antonio Espinosa de los Monteros, Miguel Antonio Rivero, Ayoze Castro, Valentín Pérez and Marisa Andrada

Special Issue
Mycobacterium avium Subspecies Paratuberculosis Infection in Ruminants—Agent, Disease, Diagnosis, and Control

Edited by
Dr. Karsten Donat and Dr. Heike Köhler

MDPI

https://doi.org/10.3390/ani13101693

animals

MDPI

Article

Morphological Assessment of Concomitant Lesions Detected in Goat Herds Naturally Infected with Paratuberculosis (Johne's Disease)

Elena Plamenova Stefanova [1,2], Óscar Quesada-Canales [1,2,*], Yania Paz-Sánchez [1,2], María José Caballero [1,2,*], María del Pino Quintana-Montesdeoca [3], Antonio Espinosa de los Monteros [1,2], Miguel Antonio Rivero [1,2], Ayoze Castro [1,2], Valentín Pérez [4,5] and Marisa Andrada [1,2]

[1] Veterinary Histology and Pathology, Institute of Animal Health and Food Safety (IUSA), Veterinary School, University of Las Palmas de Gran Canaria, 35413 Arucas, Gran Canaria, Spain; elena.plamenova101@alu.ulpgc.es (E.P.S.); yania.paz102@gmail.com (Y.P.-S.); antonio.espinosa@ulpgc.es (A.E.d.l.M.); miguel.rivero@ulpgc.es (M.A.R.); ayoze.castro@ulpgc.es (A.C.); marisaana.andrada@ulpgc.es (M.A.)

[2] Departament of Morphology, Veterinary School, University of Las Palmas de Gran Canaria, 35413 Arucas, Gran Canaria, Spain

[3] Departament of Mathematics, University of Las Palmas de Gran Canaria, 35017 Las Palmas de Gran Canaria, Gran Canaria, Spain; mariadelpino.quintana@ulpgc.es

[4] Departamento de Sanidad Animal, Facultad de Veterinaria, Campus de Vegazana, Universidad de León, 24007 León, Spain; valentin.perez@unileon.es

[5] Departamento de Sanidad Animal, Instituto de Ganadería de Montaña (CSIC-ULE), 24346 Grulleros, Spain

* Correspondence: oscar.quesada@ulpgc.es (Ó.Q.-C.); mariajose.caballero@ulpgc.es (M.J.C.)

check for updates

Citation: Stefanova, E.P.; Quesada-Canales, Ó.; Paz-Sánchez, Y.; Caballero, M.J.; Quintana-Montesdeoca, M.d.P.; Espinosa de los Monteros, A.; Rivero, M.A.; Castro, A.; Pérez, V.; Andrada, M. Morphological Assessment of Concomitant Lesions Detected in Goat Herds Naturally Infected with Paratuberculosis (Johne's Disease). *Animals* **2023**, *13*, 1693. https://doi.org/10.3390/ani13101693

Academic Editors: Karsten Donat and Heike Köhler

Received: 28 March 2023
Revised: 15 May 2023
Accepted: 17 May 2023
Published: 19 May 2023

Simple Summary: Paratuberculosis (PTB) is a well-known disease with considerable financial impact on the farm industry worldwide. Nevertheless, data regarding the assessment of naturally infected goat herds is limited. The present study describes in detail the observed gross and histological lesions detected in 39 necropsies of goats (15 vaccinated and 24 non-vaccinated) from herds with a confirmed history of PTB. PTB microscopic lesions of various grades were detected in all animals in target organs and the presence of the causative agent was confirmed using different laboratory tools. The main inflammatory findings affected the hemolymphatic, respiratory and gastrointestinal systems. The lesions were confirmed microscopically with lesser macroscopically visible alterations. Our result demonstrated that non-vaccinated animals presented more severe PTB intestinal lesions and had respiratory inflammation in all age groups studied. Those also presented a higher prevalence of ileocecal valve PTB lesions. Gastrointestinal non-PTB lesions were detected in higher number in non-vaccinated goats. Thus, histology is a powerful tool for herd diagnosis and assessment. Mainly inflammatory lesions of the respiratory and gastrointestinal tract were detected in the studied PTB-affected herds. Additionally, vaccination against PTB could play a key role in the reduction of lung and gastrointestinal inflammatory processes present in the herd.

Abstract: Paratuberculosis (PTB), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), causes significant financial losses in the ruminant industry. The aim of this study is to describe the concomitant pathological findings as well as PTB-induced lesions in 39 naturally infected goats (15 vaccinated and 24 non-vaccinated). All animals exhibited MAP-induced microscopic lesions affecting target organs, although only 62% of those were detected grossly. Mainly concomitant inflammatory pathologies were recognized affecting the hemolymphatic, respiratory and gastrointestinal systems. Non-vaccinated animals exhibited both moderate and marked granulomatous enteritis in contrast with vaccinated ones which presented mild intestinal affection. Our results demonstrate that non-vaccinated animals presented pneumonia in all age groups studied (from 12 up to >48 months old). A significantly higher prevalence of ileocecal valve PTB lesions was detected in non-vaccinated animals with pneumonic lesions ($p = 0.027$). Furthermore, a reduction of gastrointestinal non-PTB processes was described in vaccinated goats. In conclusion, a PTB infected goat herd can be affected by a wide range of concomitant pathologies, mostly inflammatory in origin. Anatomic pathology is of crucial importance for correct herd diagnosis and histopathology is an indispensable tool for lesion detection. Additionally, anti-MAP vaccination could have a beneficial effect on the reduction of respiratory and gastrointestinal non-PTB diseases.

Keywords: *Mycobacterium avium* subspecies *paratuberculosis*; goat pathology; histopathology; lesions; vaccination; concomitant pathologies

1. Introduction

Paratuberculosis (PTB), also known as Johne's disease, is a progressive emaciating disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which affects domestic and wild ruminants worldwide [1–6]. Although young animals are more susceptible to infection due to the high degree of environmental contamination, the subclinical phase is long, and the originated economic losses are significant in both cattle and the small ruminant industry [4,7–9]. Clinical-phase signs are non-specific and include a reduction in daily milk yield, weight loss, poor body condition and diarrhea [10].

PTB is a disease which is impacting European countries due to the considerable economic losses it originates and its high prevalence. Herd seropositivity of up to 65% has been reported in goat and sheep herds in Germany and 62.9% in France [11,12]. In Italy, various reports highlight the need for the implementation of control strategies and viable diagnostic tools for PTB early detection [13,14]. In Spain, goat farming is one of the biggest pillars of the national livestock breeding industry since the country counts for 23% of EU's goat population (EUROSTAT 2021). Goat farms represent the primary livestock breeding sector in the Canary Islands, with 202.887 heads and 1289 dairy farms distributed as follows: 478 on Gran Canaria, 224 on Tenerife, 212 on Fuerteventura, 129 on La Palma, 117 on Lanzarote, 82 on El Hierro and 47 on La Gomera (ISTAC 2020). The archipelago has the fourth-largest goat population in Spain (Eurostat 2020), composed mainly of certified autochthonous endangered breeds (Orden APM/26/2018). In continental Spain, considerable economic losses due to goat PTB have been reported, and it is considered a widespread problem for the whole national territory with herd seroprevalence of 87.5% in southern Spain, 46% in Huelva region, 44% in Madrid and 56% in Avila, although data about the prevalence of the disease in the Canary Islands is limited [8,15].

Gross lesions in animals with PTB observed during necropsy are usually confined to the intestinal mucosa and the draining lymph nodes [6,16,17]. The ileocaecal valve mucosa can appear diffusely thickened and folded into transverse rugae, the crests of which may be congested. The mesenteric (MS) and ileocecal lymph (IC) nodes (LNs) are diffusely enlarged, edematous and pale (lymphadenomegaly, LAM). Lymphangitis can be detected in the mesentery. In some mild cases, lymphangitis is the only gross lesion specific enough to justify a presumptive PTB gross diagnosis [6,16,18]. Other commonly associated macroscopic findings are loss of muscle mass, serous atrophy of fat depots, intermandibular oedema, fluid effusion in cavities, with the presence of mineralization plates and intimal fibrosis in the thoracic aorta [6,16,18].

The main histopathological lesions associated with MAP-infection include transmural granulomatous enteritis, mesenteric and ileocecal lymph nodes granulomatous lymphadenitis and lymphangitis [6,16–18]. Villi can present moderate to marked atrophy with infiltrate of epithelioid macrophages and a variable number of Langhans-type multinucleated giant cells focally or diffusely distributed along the lamina propria, submucosa muscular layer or serosa of the intestine. The lesions observed in lymph nodes can vary from multiple foci of epithelioid macrophages up to well-formed encapsulated granulomas with central necrosis [6,16–18].

Although PTB is a major livestock farming issue of worldwide distribution, there may be a variety of other different diseases co-existing in naturally infected herds. The prevalence of the different diseases varies among the age groups present in the herd, the breed of the animals, the herd size, environmental factors as well as the variations in the pathogens present in each farm [8,15,19–21]. Respiratory, gastrointestinal, lymphatic and hepatic pathologies stand out among the main lesions detected in small ruminant herds worldwide [19,22,23].

Respiratory diseases represent 5.6% of the most common diseases in small ruminants affecting both individuals or groups, resulting in considerable financial losses due to decreased meat, milk and wool production along with a reduced number of offspring [24,25]. The etiology is multifactorial, involving mainly bacteria and viruses, and secondary infections are common. The lung can present lesions varying from acute bronchopneumonia up to severe chronic lung involvement [5,24,25].

Gastrointestinal affection, besides PTB-induced lesions, can be associated with diarrhea and weight loss due to viral, bacterial and parasitic infections. Acute lesions can vary from marked hyperemia of the intestinal mucosa up to fibrino-haemorrhagic and even necrotizing enteritis affecting both the small and large intestines. Subacute and chronic cases can exhibit multifocally distributed lesions, such as abscesses affecting the ileum, caecum and colon [6,19,22,23].

Lymphadenopathy is also considered a major clinical finding in many important infectious goat diseases. LNs are the primary disease location in cases of *C. pseudotuberculosis* induced caseous lymphadenitis [5,26]. Acute lymphadenitis, on the other hand, is a common finding in regional LNs associated with organs affected by bacterial infection [5,19,26].

Specific liver lesions are mainly of inflammatory origin (hepatitis) [5]. In adult animals, abscesses are commonly observed in association with *Trueperella pyogenes* or *Fusobacterium necrophorum* infection. Necrotizing hepatitis caused by *Clostridium* spp. is also a common finding [5,27,28].

Anatomopathological examination (gross and histopathological findings) is crucial to adequate herd diagnosis [16,18,29]. For correct assessment and confirmation of the suspected diagnosis or new insights on the investigated disease problem in the herd, all possible necropsies following a systematic procedure should be performed as a useful adjunct to clinical examination [5,19,22,25].

The present study aims to provide a morphological classification of a variety of MAP-induced lesions in naturally infected animals as well as to recognize the different concomitant pathological findings in 39 female majorera goats with histopathological PTB lesions detected during a routine postmortem examination.

2. Materials and Methods

2.1. Study Design

A total of 39 adult goats with histological PTB lesions were included in the study. All animals were submitted with a presumption of a field PTB diagnosis and were examined by the Anatomic Pathology Diagnostic Service of the Veterinary School of the University of Las Palmas de Gran Canaria. The animals were submitted from 8 farms (F1, F2, F3, F4, F5, F6, F7 and F8) in the Canary Islands with a confirmed history of PTB. The on-farm confirmation protocol included serological ELISA immunoassay (PARACHEK® 2 Kit, Thermo Fisher Scientific, Waltham, MA, USA), bacteriological culture of environmental samples and subsequent PCR confirmation, postmortem examination and compatible clinical signs detection. A total of 15/39 animals from 3 of the farms were vaccinated against MAP (Table 1). All goats selected presented histological lesions compatible with PTB including granulomatous enteritis (GE) with the presence of focal to multifocal/diffuse transmural accumulations of epithelioid cells and lymphocytes typically accompanied by Langhans-type multinucleated giant cells (MGCs) and/or granulomatous lymphadenitis (GLA) of the mesenteric lymph nodes (MS LNs) characterized by the infiltration of epithelioid macrophages and/or Langhans-type MGCs.

Table 1. Number of vaccinated (V) and non-vaccinated (nV) goats included in the study by farm and age range.

Farm	Age Range (Months)						Total
	(0–12]	(12–24]	(24–36]	(36–48]	>48	Unknown	
F1	1V	2V	4V	-	-	1V	8V
F2	-	5V + 1nV	1V	-	-	-	6V + 1nV
F3	-	1V + 7nV	-	-	-	-	1V + 7nV
F4	-	5nV	1nV	-	-	-	6nV
F5	-	-	1nV	-	3nV	-	4nV
F6	-	-	2nV	-	-	-	2nV
F7	-	-	-	1nV	1nV	-	2nV
F8	-	-	1nV	1nV	-	-	2nV
Total (n = 39 [1])	1V	8V + 13nV	5V + 5nV	2nV	4nV	1V	15nV + 24nV

V, vaccinated; nV, non-vaccinated. [1] Data about the age of 38/39 animals was available. All animals included in the study presented histological lesions compatible with PTB, including granulomatous lymphadenitis of the mesenteric lymph nodes and/or granulomatous enteritis.

According to the local legislation (Decree 51/2018 del 23 de abril), caprine vaccination against MAP is only permitted after "tuberculosis-free" status confirmation and PTB diagnostic confirmation since the archipelago was declared "officially free" of bovine tuberculosis (TB) in 2017. The vaccination protocols in the 8 farms studied included commercial vaccines against *E. coli*, *Clostridium* spp. (enterotoxemia), *Chlamydia* spp. (abortion) and *Pasteurella* spp. (bronchopneumonia) which were applied following the manufacturer's recommendations.

2.2. Anti-MAP Vaccine

Gudair® is a 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. It had been administered once subcutaneously as per the manufacturer's instructions in the three farms applying PTB vaccination enrolled in this study.

2.3. Sample Collection and Processing for Histolgical Examination

Necropsies were performed following the protocol proposed by King et al., 2014 [29]. Lymph nodes and intestines were sampled for histology according to the recommendations of the national bovine tuberculosis eradication program protocol 2021 (MAPA, 2021) as follows: mesenteric (MS), ileocecal (IC), retropharyngeal (RPh), prescapular (PE) and mediastinal (MD) lymph nodes (LNs) and ileocecal valve region (ICV). Samples from representative organs (lungs and trachea, liver, kidneys and urinary bladder, heart, LNs, spleen, intestine, forestomaches and abomasum, uterus and ovaries, mammary gland, skeletal muscle and brain) were systematically collected for histological examination.

All tissue samples were fixed in 10% buffered formalin, embedded in paraffin and processed routinely, sectioned at 5 µm and stained with hematoxylin/eosin (HE). Additionally, selected tissue sections of 4 µm were stained with the Ziehl–Neelsen (ZN) technique for detecting acid-fast bacilli (AFB).

2.4. Gross Evaluation

All macroscopic lesions were recorded and described in terms of location, color, size, shape, consistency and number or percent of involvement of the affected organ and a presumed morphological gross diagnosis was established by an experienced pathologist.

2.5. Histological Evaluation

All histopathological findings were detailed and organized according to the system affected.

Two grading systems were applied to evaluate the PTB-compatible histological lesions. MS LNs lesions were classified as 0 (no lesions), I (initial), II (solid), III (minimal necrosis) and IV (necrosis and mineralization) as per Wangoo et al., 2005, grading score described for tuberculosis lymph node granulomas [30]. The lesions observed in the lamina propria (LP) and the Peyer's patches (PPs) of the ICV were graded separately in terms of severity (mild, moderate and marked) and distribution (focal, multifocal and diffuse) using the grading system proposed by Krüger et al., 2015 [18]. Severity was graded as mild when only small circumscribed lymphocytic or 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 [18]. Lesion distribution was considered focal when up to 3 distinct granulomatous infiltrates were observed per section; multifocal when more than 3 granulomatous distinct infiltrates per section were seen; and diffuse when granulomatous infiltrate was present throughout the whole section [18].

2.6. MAP Confirmation by Immunohistochemistry

Selected sections (3 µm) from MS LNs and ICV were immunohistochemically labelled with a polyclonal anti-MAP in-house antibody kindly provided by Dr. V. Perez, University of León, León, Spain. A polymer-based detection system (EnVision®System Labelled Polymer-HRP; Dako, Glostrup, Denmark) was employed, following the manufacturer's instructions. Subsequently, immunolabelling was developed with a commercial solution of 3,3′diaminobenzidine (DAB) (K3468; Dako, Glostrup, Denmark). The slides were counterstained with Harris´ hematoxylin and mounted in a hydrophobic medium.

The number of mycobacteria per section was graded using a scoring system described by Krüger et al., 2015 [18], as follows: none (<2 labelled bacteria per section), single (mycobacteria in <20% epithelioid cells and/or MGCs; single/few bacteria per cell or foci of granular labelling, predominantly <21 mm in diameter), few (mycobacteria in 20% to 50% epithelioid cells and/or MGCs; on average, 1 to 10 bacteria per cell or foci of granular labeling predominantly >21 mm in diameter) and many (mycobacteria in >50% to 75% epithelioid cells and/or MGCs; on average >10 bacteria per cell, with up to 50% of cells containing countless bacteria).

2.7. qPCR and Bacterial Culture for MAP Identification

In order to confirm MAP infection, frozen tissue samples from MS LNs and ICV were submitted to external laboratories for real-time polymerase chain reaction (qPCR) and bacterial culture. Frozen tissue samples from 29/39 animals were available for examination. Molecular biology qPCR targeting IS900 gene was performed on samples 13/29 animals using EXOone qPCR kit (EXOPOL SL, Zaragoza, Spain). Samples were considered positive when Cq $\leq$ 38. Tissues from 16/29 animals were examined by bacterial culture as part of the national tuberculosis eradication and surveillance program. Samples from 3/29 animals were submitted to both qPCR and bacterial culture exams. The culture analysis was carried out by the national infectious diseases reference laboratory of VISAVET, Health Surveillance Centre, Madrid, Spain. The qPCR was performed by a private external laboratory.

2.8. Statistical Analysis

An observational cross-sectional study was carried out. The prevalence of macroscopic and microscopic pathological findings was calculated separately in each organic system affected. Lesion type prevalence was also measured in each system affected analyzing gross and histological findings separately.

Statistical analysis of data was performed by IBM SPSS Statistics 27 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA: IBM Corp). Categorical variables were summarized using percentages and relative or absolute frequencies. The ages of the studied goats were categorized in the following groups: (0–12] months, (12–24] months, (24–36] months, (36–48] months and >48 months. Chi-square test was used to contrast the association between two categorical variables. To analyze the association between two ordinal scale variables, Kendall's Tau-b test was applied. Numerical variables were summarized using the mean, standard deviation (SD), median and interquartile range (IQR). A Shapiro–Wilk test was applied to analyze the sample normality. A non-parametric Mann–Whitney U test was used to compare two independent samples when the condition for normality of the numerical variable was not met. Agreement between qualitative results obtained by two measurement procedures was calculated using the kappa coefficient (κ). The results were considered statistically significant if the p value < 0.05.

3. Results

3.1. Necropsy Findings

All animals studied exhibited poor body conditions characterized by severe emaciation, protrusion of lumbar vertebrae and easily palpable transverse processes, muscle mass loss and a reduction of visceral fat deposits.

Data about the age of the animals was available in 38/39 cases. The studied animals were between 7 and 121 months of age, with a mean of 29 months, median of 16.50 months, SD of 30.2 months and IQR of 16 months. The ages of the studied goats were categorized in the following groups: (0–12] months, (12–24] months, (24–36] months, (36–48] months and >48 months in which 1, 21, 10, 2 and 4 animals were included, respectively.

PTB granulomatous lesions were observed in 61.5% (24/39) of the animals affecting the MS LNs (Figure 1a and Table 2). PTB intestinal gross lesions were described in 7/39 (17.9%) cases consisting in the thickened, corrugated mucosal surface of the small intestine and the ICV region (Figure 1b and Table 2).

A significant difference was observed between the month of age of the animals and the presence of macroscopic PTB granulomatous lesions affecting the MS LNs (p = 0.022). Once the ages of the vaccinated and non-vaccinated groups were compared in relation with PTB induced lesions in MS LNs, significant variation was observed only in the non-vaccinated group (p = 0.029) in contrast with the vaccinated where the animals were mostly between 12 and 24 months (p = 0.471).

Granulomatous lymphadenitis was also detected in the RPh (2/39; 5.1%) and the mammary (MA) (2/39; 5.1%) LNs. Those LNs appeared firm and enlarged, with a thickened capsule. The cut surface revealed the partial to complete loss of the architecture, replaced by multiple white, firm poorly demarcated areas which often presented central caseous necrosis and mineralization.

Lymph nodes were one of the most affected organs (80%; 31/39). Lymphadenomegaly was detected in 64% (25/39) of the animals affecting mainly the MS (21/25), IC (9/25) and MA (9/25) LNs (Figure 1c). Inflammatory lesions (lymphadenitis) were seen in 57% (22/39), affecting different LNs.

Suppurative lymphadenitis (SLA) was observed in the PE (6/39; 15.4%) and MD (3/39; 7.7%) LNs. Those LNs appeared enlarged, soft and hyperemic with thick fibrous well-formed capsules. On the cut surface, the parenchyma bulged and exudated variable amounts of blood, pus or lymph.

Caseous lymphadenitis (CLA) characterized grossly by the classic "cheesy gland" appearance parenchyma affected the RPh (2/39; 5.1%), MD (3/39; 7.7%) and MA (2/39; 5.1%) LNs. Those LNs were large and firm, exudating variable amounts of pasty yellowish material (pus) which completely substituted the organ's parenchyma (Figure 1d).

Abscess formation characterized by the complete substitution of the normal lymph node parenchyma by large amount of yellow-green pus surrounded by a thick fibrous capsule was seen affecting the RPh (3/39; 7.7%), PE (4/39; 10.3%), MD (2/39; 5.1%) and MA (1/39; 2.6%) LNs.

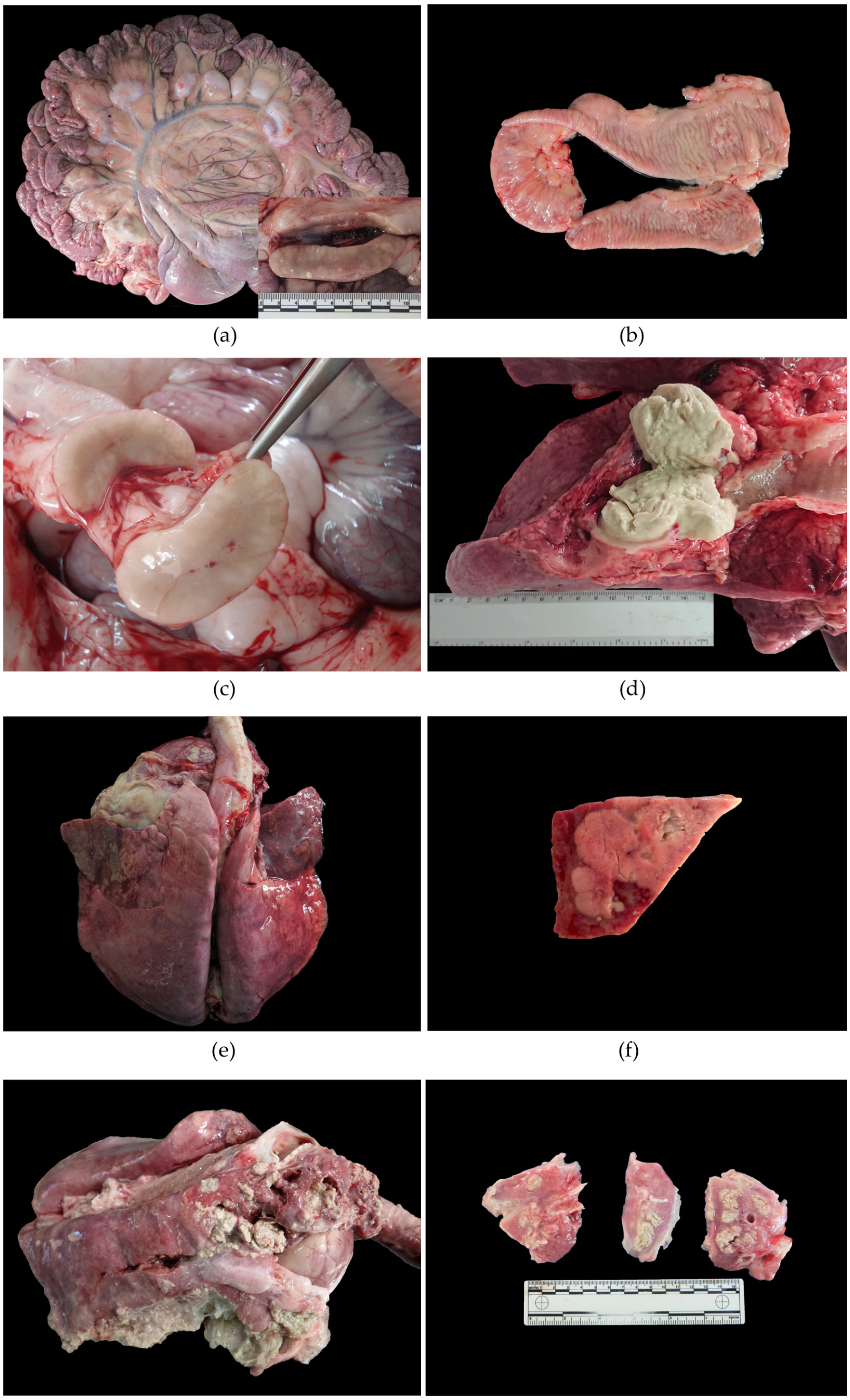


Figure 1. Main gross findings in 39 naturally infected goats with PTB lesions. (**a**) Severe lymphadenomegaly (LAM) affecting the mesenteric lymph nodes (MS LNs); inset: MS LNs with white calcified granulomas on the cut surface. (**b**) Jejunum. Diffuse thickening of the intestinal mucosa folded into transverse rugae, characteristic of PTB. (**c**) Lymphadenomegaly (LAM) in an MS LN. Enlarged lymph node with uniform cut surface. (**d**) Caseous lymphadenitis (CLA) affecting mediastinal lymph nodes (MD LNs) with abundant caseous material replacing the parenchyma completely. (**e**) Cranioventral bronchopneumonia (CBP) characterized by cranioventral dark red consolidation of lung lobes. (**f**) CBP lung section showing whitish areas of necrosis and purulent exudate. (**g**,**h**) Severe CBP affecting 70% of the lung with abundant purulent exudate oozing from airways.

Table 2. Summary of MAP-induced morphological findings and lung pneumonic lesions in 39 naturally infected goats.

			Age Range (Months)								**Total**		
			(0–12]	**(12–24]**		**(24–36]**		**(36–48]**	**>48**	**Unknown**	**V** *n* **= 15**	**nV** *n* **= 24**	*n* **= 39**
Vaccination			**V** *n* **= 1**	**V** *n* **= 8**	**nV** *n* **= 13**	**V** *n* **= 5**	**nV** *n* **= 5**	**nV** *n* **= 2**	**nV** *n* **= 4**	**V** *n* **= 1**			
Gross PTB lesions	MS LNs	Yes	1 (100%)	6 (75%)	8 (61.5%)	4 (80%)	2 (40%)	1 (50%)	1 (25%)	1 (100%)	12 (80%)	12 (50%)	24 (61.5%)
		No	-	2 (25%)	5 (38.5%)	1 (20%)	3 (60%)	1 (50%)	3 (75%)	-	3 (20%)	12 (50%)	15 (38.5%)
	ICV	Yes	-	3 (37.5%)	1 (7.7%)	3 (60%)	-	-	-	-	6 (40%)	1 (4.2%)	7 (17.9%)
		No	1 (100%)	5 (62.5%)	12 (92.3%)	2 (40%)	5 (100%)	2 (100%)	4 (100%)	1 (100%)	9 (60%)	23 (95.4%)	32 (82.1%)
Histological PTB lesions	MS LNs (Severity grade)	I	-	-	3 (23.1%)	-	1(20%)	-	3 (75%)	-	-	7 (29.2%)	7 (17.9%)
		III	-	-	2 (15.4%)	-	-	-	-	-	-	2 (8.3%)	2 (5.1%)
		IV	1 (100%)	8 (100%)	7 (53.8%)	5 (100%)	3 (60%)	1 (50%)	1 (25%)	1 (100%)	15 (100%)	12 (50%)	27 (69.2%)
		Subtotal	1 (100%)	8 (100%)	12 (92.3%)	5 (100%)	4 (80%)	1 (50%)	4 (100%)	1 (100%)	15 (100%)	21 (87.5%)	36 (92.3%)
		No	-	-	1 (7.7%)	-	1 (20%)	1 (50%)	-	-	-	3 (12.5%)	3 (7.7%)
	ICV LP (severity)	Mild	-	5 (62.5%)	3 (23.1%)	4 (80%)	-	1 (50%)	1 (25%)	-	9 (60%)	5 (20.8%)	14 (35.9%)
		Moderate	-	-	1 (7.7%)	-	1 (20%)	1 (50%)	1 (25%)	-	-	4 (16.7%)	4 (10.3%)
		Marked	-	-	-	-	1 (20%)	-	1 (25%)	-	-	2 (8.3%)	2 (5.1%)
		Subtotal	-	5 (62.5%)	4 (30.8%)	4 (80%)	2 (40%)	2 (100%)	3 (75%)	-	9 (60%)	11 (45.8%)	20 (51.3%)
		No	1 (100%)	3 (37.5%)	9 (69.2%)	1 (20%)	3 (60%)	-	1 (25%)	1 (100%)	6 (40%)	13 (54.2%)	19 (48.7%)
Histological pneumonic lesions	Yes		-	7 (87.5%)	10 (76.9%)	-	4 (80%)	1 (50%)	2 (50%)	1 (100%)	8 (53.3%)	17 (70.8%)	25 (64.1%)
	No		1 (100%)	1 (12.5%)	3 (23.1%)	5 (100%)	1 (20%)	1 (50%)	2 (50%)	-	7 (46.7%)	7 (29.2%)	14 (35.9%)

PTB, paratuberculosis; MS LNs, mesenteric lymph nodes; ICV LP, ileocecal valve lamina propria; V, vaccinated; nV, non-vaccinated.

The respiratory tract was affected in 17/39 (43.6%), mainly by bronchopneumonia (CBP) (16/39; 41%) characterized by cranioventral consolidation of the lungs, affecting at least 30% of the parenchyma, with dark red to maroon discoloration and an oedematous cut surface (Figure 1e,f). In some cases, purulent or catarrhal material oozed from the small airways. Fibrous adhesions between the parietal and visceral pleura (fibrous pleuritis) were observed in 5/39 (12.8%), along with CBP. The presence of fibrin strands or vails on the pleural surface (fibrinous pleuritis) along with CBP was described in seven (17.9%) (Figure 1g,h). No association with PTB macroscopic lesions was observed (p = 0.792) and no differences between the ages of the animals affected were demonstrated (p = 0.854).

The gastrointestinal tract exhibited gross lesions not categorized as PTB, in particular, a nonspecific diffusely thickened appearance in 10/39 animals (25.6%) and hemorrhagic enteritis in one animal (2.6%). The lesions detected had statistically significant associations with PTB gross lesions in MS LNs only in the non-vaccinated group (p = 0.013).

The liver exhibited a uniform pale yellow color, round edges and friable texture (steatosis) in 7/39 (17.9%) of the cases and multifocal, white, firm, well demarcated areas of capsular and/or subcapsular fibrosis in 6/39 (15.4%). One animal presented multifocal well-demarcated mineralization.

The heart was affected in 13% (5/39) of the cases by a variety of lesions including hydropericardium (2/39; 5.1%), fibrous pericarditis (2/39; 5.1%) and petechial haemorrhages on the pericardial surface (2/39; 5.1%).

Kidneys were affected in 5% (2/39) of the cases, presenting multiple small white nodules of up to 1 cm diameter throughout the cortex, consistent with nonsuppurative interstitial nephritis.

A summary of the main gross findings affecting the 39 goats included in the study can be found as Appendix A (Table A1).

3.2. Microscopical Findings

Lesions consistent with PTB were observed in 100% of the cases (granulomatous enteritis and/or granulomatous lymphadenitis of the MS LNs). In affected MS LNs (92.3%, 36/39), grade IV granulomas were described in 27/36 (75%) of the animals (Figure 2). Although, severe granuloma formation (grade IV) was not always the main PTB lesions detected in affected LNs. In fact, in 16/36 (44%) of the cases, grade I multifocal microgranulomas constituted the predominant lesion type in the examined samples (Figure 2a). All vaccinated goats (15/15, 100%) exhibited grade IV lesions in contrast with the non-vaccinated ones where 7/24 (29.2%) and 2/24 (8.3%) animals only presented grade I and grade III lesions, respectively (Table 2). The PPs could be evaluated in 33 animals, 70% (23/33) of which presented PTB-compatible lesions of mild severity (46%, 15/33) and multifocal distribution (55%, 18/33). The LP was affected in 51.3% (20/39) of the cases, mainly by mild lesions (35.9%, 14/39) with multifocal distribution (48.7%,19/39) (Figure 2b and Table 2). In the vaccinated group, 9/15 (60%) animals were affected, presenting mild ICV lesions affecting the LP. In contrast, the non-vaccinated PTB affected goats had moderate and marked lesions in 4/24 (16.7%) and 2/24 (8.3%) of the cases, respectively. No significant differences were described between the age groups of the animals studied (p = 0.085), as well as between the medians of the vaccinated (14 months) and non-vaccinated (17 months) goats (p = 0.691).

Reactive hyperplastic lesions consisted in follicular hyperplasia with larger follicles with a paler germinal center containing an increased numbers of apoptotic lymphocytes and tingible body macrophages were observed in 53% of the animals affecting different lymph nodes (Figure 2c).

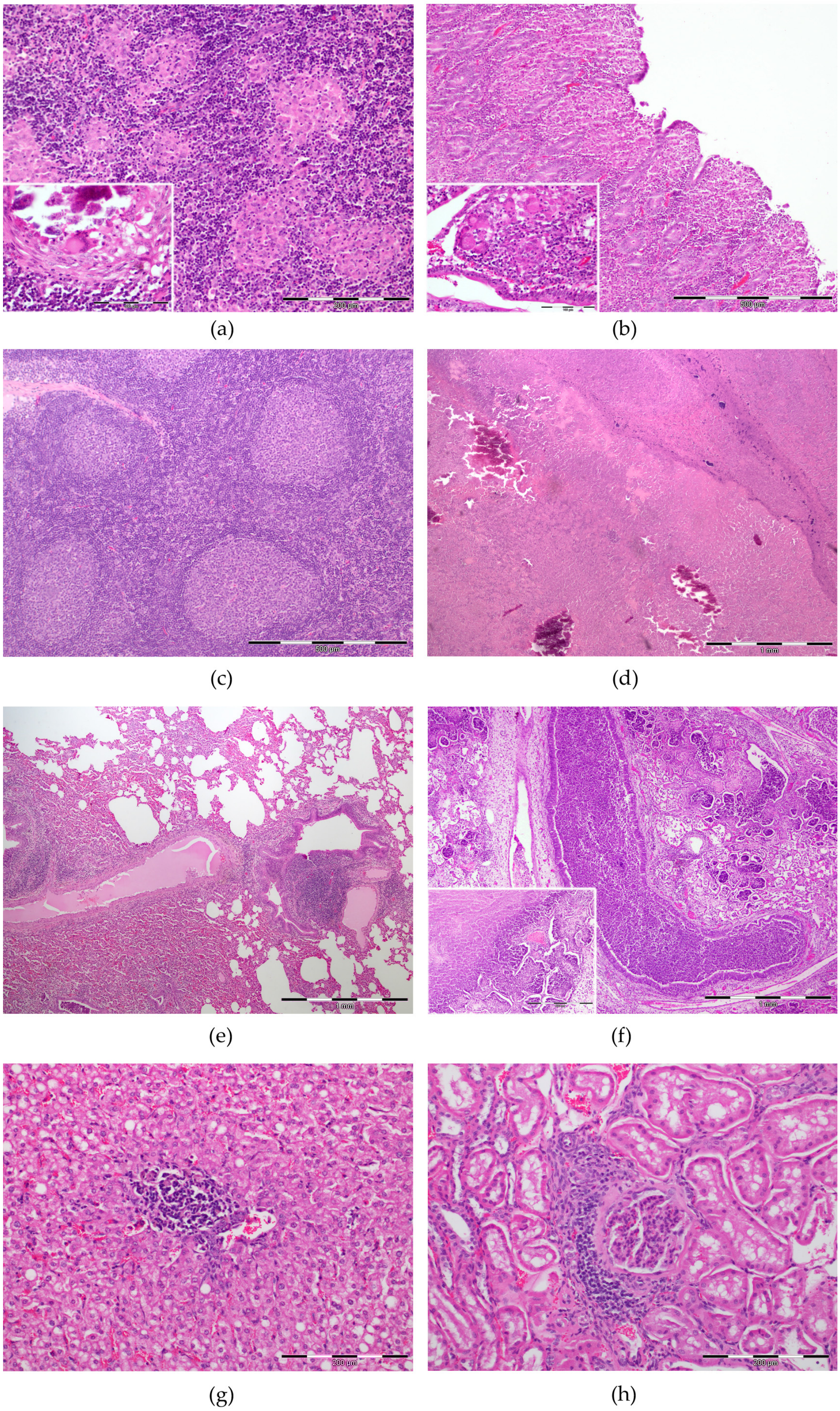


Figure 2. Main histopathological findings in 39 naturally infected goats with PTB lesions. (**a**) Mesenteric lymph node (MS LN), scale: 200 µm: multifocal aggregates of epithelioid macrophages

inset (scale: 100 µm): Langhans-type multinucleated giant cells (MGCs) in a calcified granuloma. (**b**) Jejunum (scale: 500 µm): granulomatous enteritis formed by groups of macrophages and diffuse infiltration of lymphocytes; inset (scale: 100 µm): Langhans-type MGCs with epithelioid macrophages aggregates in intestinal villi tip. (**c**) Lymph node (LN) (scale: 500 µm): reactive lymphoid hyperplasia characterized by large lymphoid follicles with prominent germinal centers. (**d**) LN (scale: 1 mm): caseous lymphadenitis/suppurative lymphadenitis (CLA/SLA) with vast areas of lytic necrosis with mineralization. (**e**) Lung (scale: 1 mm): bronchointerstitial pneumonia (BIP) characterized by proliferation of type II pneumocytes, associated lymphoid tissue and thickening of the alveolar septa by lymphoid infiltration. (**f**) Lung (scale: 1 mm): severe fibrinonecrotizing bronchopneumonia (FNBP) with degenerated neutrophils, fibrin and necrotic debris filling bronchioles and alveoli and large areas of coagulative necrosis (inset, scale: 500 µm). (**g**) Liver (scale: 200 µm): perivascular lymphohistiocytic hepatitis characterized by foci of lymphocytes and occasional macrophages and mild steatosis. (**h**) Kidney, cortex (scale: 100 µm): interstitial nephritis with lymphohistiocytic infiltration expanding the interstitium.

LNs were mainly affected by CLA and SLA (Figure 2d). CLA was characterized by variable-sized areas of lytic necrosis admixed with degenerated and viable neutrophils surrounded by epithelioid macrophages, lymphocytes and plasma cells, and a thick capsule of fibrous connective tissue. On the other hand, SLA LNs exhibited marked hyperaemia with diffuse infiltration of a moderate to large number of neutrophils, vast areas of necrosis and mineralization. The affected lymph nodes were the RPh, PE, MD and MA with lesions observed in 8/39 (20.5%), 4/39 (10.3%), 8/39(20.5%) and 4/39 (10.3%) of the cases, respectively. Abscess formation was confirmed only grossly and was not included in the histological analysis. The GLA described grossly in RPh and MA LNs were histologically classified as SLA/CLA.

Various organs also showed diverse and different lesions, mainly the respiratory tract, digestive tract, liver, cardiovascular system and kidneys.

The main lesions observed in the respiratory tract were inflammatory pneumonia processes (64.1%, 25/39) (Table 2). Necrotic-suppurative bronchopneumonia characterized by the presence of viable and degenerated neutrophils, fibrin and cell debris, filling bronchioles and alveoli, admixed with areas of necrosis and haemorrhage was detected in 12/39 animals (30.8%) (Figure 2f). Lymphohistiocytic interstitial pneumonia (IP) with increased number of lymphocytes and histiocytes thickening the alveolar septa was diagnosed in 6/39 (15.4%) of the cases. Bronchointerstitial pneumonia (BIP) was described in 3/39 animals (7.7%) and included bronchiolar necrosis, diffuse alveolar damage, thickening of the alveolar septa by lymphoid infiltration and hyperplasia of type II pneumocytes, perivascular lymphoid infiltration and hyperplasia of associated lymphoid tissue (Figure 2e). In 4/39 cases (10.3%), the lungs presented only fibrous pleuritis. In the vaccinated and non-vaccinated animals, pneumonia lesions were detected in 8/15 (53.3%) and 17/24 (70.8%), respectively (Table 2).

Differences between the age groups of vaccinated and non-vaccinated animals were detected regarding the presence of microscopic lung inflammation. Most of the vaccinated animals with histological lesions had between 12 and 24 months of age ($p = 0.035$) (Figure 3a). Those lesions in the non-vaccinated goats were distributed in ages from 12 months up to >120 months, with no significant differences between the number of animals in each age range ($p = 0.677$) (Figure 3b).

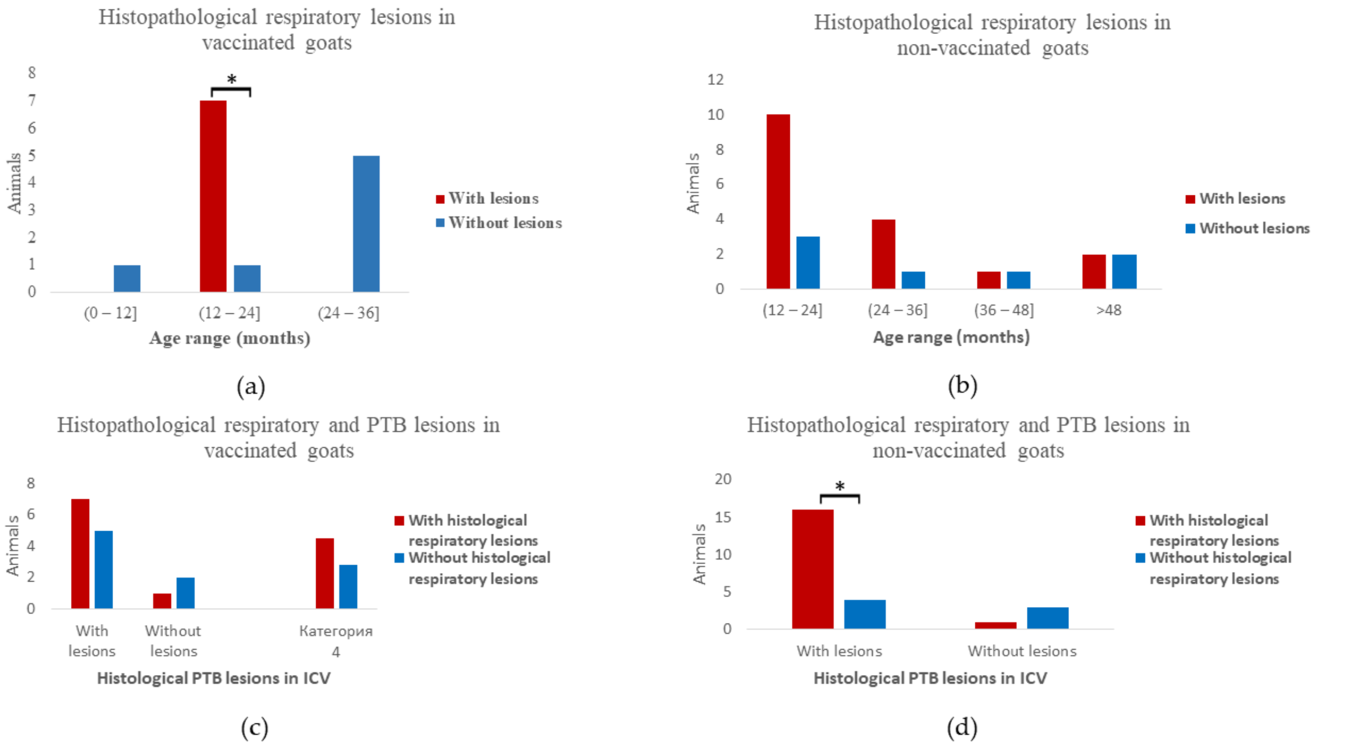


Figure 3. Associations between age groups, histopathological respiratory lesions and PTB histological lesions affecting the ileocecal valve (ICV) in vaccinated and non-vaccinated goats. (**a**) Distribution of vaccinated goats in different age groups in relation with presence of histopathological lesions of the respiratory tract. * $p = 0.035$ animals affected were mainly >12 < 24 months of age. (**b**) Distribution of non-vaccinated goats in different age groups in relation with presence of histopathological lesions of the respiratory tract. (**c**) Associations between the presence of histopathological respiratory lesions and histopathological PTB lesions in the ICV of vaccinated goats. (**d**) Associations between the presence of histopathological respiratory lesions and histopathological PTB lesions in the ICV of non-vaccinated goats. * $p = 0.027$ more non-vaccinated animals with PTB lesions affecting the ICV presented histological respiratory lesions.

In relation to the presence of histological inflammation affecting the respiratory tract (25/39), grade IV granulomas in the MS LNs were present in 16/25 (64%) animals ($p = 0.145$). Furthermore, significantly more non-vaccinated animals with MAP-induced lesions in the ICV presented lung inflammation ($p = 0.027$) (Figure 3d). No such association was described in the vaccinated group ($p = 0.438$) (Figure 3c).

Regarding the digestive system, lymphoplasmacytic (LPE) and eosinophilic enteritis (EE) were the main findings observed. LPE, characterized by a diffuse discrete-moderate number of lymphocytic mucosal infiltrate, was detected in 1/39 (2.6%) of the animals. EE characterized by eosinophils with a smaller number of globular leucocytes and plasma cells, infiltrating and expanding the intestinal mucosa was described in 2/39 (5.1%). The presence of coccidia of *Eimeria* spp. was confirmed in one animal with EE. Mild lymphohistiocytic abomasitis or gastric non-perforated ulcers were described in 4/39 (10.3%) of the animals.

There was no difference between the ages of the eight animals affected ($p = 0.430$). Seven (7) of those were non-vaccinated and only one was vaccinated ($p = 0.090$). Five (5) of the non-vaccinated affected goats (71%) exhibited grade IV granulomas affecting the MS LNs ($p = 0.170$).

The most frequent hepatic finding was lymphohistiocytic hepatitis (64.1%, 25/39), characterized by multiple foci of lymphocytes and occasional macrophages, mainly with a periportal distribution (Figure 2g). Hepatocellular steatosis with hepatocytes presenting one large or multiple variably sized discrete intracytoplasmic vacuoles, frequently displacing and compressing the nucleus to the periphery, was also observed (11/39, 28.2%).

Kidneys presented non-suppurative interstitial nephritis, mostly affecting the cortex and to a lesser extent, the medulla, with the diffuse infiltration of lymphocytes and occasional plasma cells and macrophages, interstitial fibrosis and tubular atrophy in 25.6% (10/39) of the cases (Figure 2h).

The heart was predominantly affected by the presence of apicomplexan schizont, containing large numbers of oval basophilic bradyzoites, compatible with *Sarcocystis* spp., expanding cardiomyocytes (9/39, 23.1%). Non-suppurative myocarditis characterized by multifocal lymphocytic infiltration and occasional myofiber degeneration was also observed (3/39, 7.7%). Fibrous pericarditis was confirmed in 3/39 (7.7%).

A summary of the histological lesions detected in the 39 animals studied can be found as Appendix A (Table A1).

3.3. MAP Identification (Immunohistochemistry, Ziehl–Neelsen, Bacterial Culture and qPCR)

In all cases included in the study, MAP positivity was confirmed in MS LNs and/or VIC by at least one of the techniques used. Positivity by both IHC and ZN was detected as cytoplasmic infiltrate in epithelioid macrophages and giant cells. In the case of IHC, immunoreaction was seen as brown cytoplasmic granules and as red bacilli by ZN (Figure 4). In MS LNs, results for ZN and IHC staining suggested MAP positivity in 82.1% (32/39) and 84.6% (33/39) of the cases, respectively (Table 3). The LP was positive by ZN in 53.9% (21/39) and in 59% (23/39) by IHC. The PPs were present in 33/39 of the cases and AFB were observed in 61% (20/33) by ZN and MAP-antigens were detected in 67% (22/33) by IHC. Moderate correlation between the detection capacity of ZN and IHC in MS LNs (κ = 0.537), ICV LP (κ =0.584) and ICV PP (κ = 0.609) was demonstrated. The main difference between the two techniques was observed in the intensity of the reaction which was stronger with IHC.

Table 3. Summary of Ziehl–Neelsen (ZN) and immunohistochemistry (IHC) results for MAP detection in 39 naturally infected goats.

			Age Range (Months)								**Total**		
			(0–12]	**(12–24]**		**(24–36]**		**(36–48]**	**>48**	**Unknown**			
Vaccination			**V** *n* = 1	**V** *n* = 8	**nV** *n* = 13	**V** *n* = 5	**nV** *n* = 5	**nV** *n* = 2	**nV** *n* = 4	**V** *n* = 1	**V** *n* = 15	**nV** *n* = 24	*n* = 39
MS LN	ZN	(+)	1 (100%)	6 (75%)	11 (84.4%)	4 (80%)	4 (80%)	2 (100%)	3 (75%)	1 (100%)	12 (80%)	20 (83.3%)	32 (82.1%)
		(−)	-	2 (35%)	2 (15.4%)	1 (20%)	1 (20%)	-	1 (25%)	-	3 (20%)	4 (16.7%)	7 (17.9%)
	IHC	(+)	1 (100%)	7 (87.5%)	10 (76.9%)	5 (100%)	4 (80%)	1 (50%)	4 (100%)	1 (100%)	14 (93.3%)	19 (79.2%)	33 (84.6%)
		(−)	-	1 (12.5%)	3 (23.1%)	-	1 (20%)	1 (50%)	-	-	1 (6.7%)	5 (20.8%)	6 (15.4%)
ICV LP	ZN	(+)	-	4 (50%)	7 (53.8%)	3 (60%)	3 (60%)	-	4 (100%)	-	7 (46.7%)	14 (58.3%)	21 (53.9%)
		(−)	1 (100%)	4 (50%)	6 (46.2%)	2 (40%)	2 (40%)	2 (100%)	-	1 (100%)	8 (53.3%)	10 (41.7%)	18 (46.1%)
	IHC	(+)	1 (100%)	5 (62.5%)	5 (38.5%)	3 (60%)	4 (80%)	1 (50%)	4 (100%)	-	9 (60%)	14 (58.3%)	23 (59%)
		(−)	-	3 (37.5%)	8 (61.5%)	2 (40%)	1 (20%)	1 (50%)	-	1 (100%)	6 (40%)	10 (41.7%)	16 (41%)

MS LN, mesenteric lymph node; ICV LP, ileocecal valve lamina propria; ZN, Ziehl–Neelsen; IHC, immunohistochemistry; (+) positive; (−) negative; V, vaccinated; nV, non-vaccinated.

A total of 29/39 animals were examined for MAP confirmation by qPCR (13/29) and/or bacterial culture (16/29). Four animals (4/16, 25%) tested positive by bacterial culture and 9/13 (69.2%) by qPCR. Samples from 3/29 animals were tested with both techniques, and all were identified as negative by bacterial culture and as positive by qPCR. The same three animals were positive by both ZN and IHC.

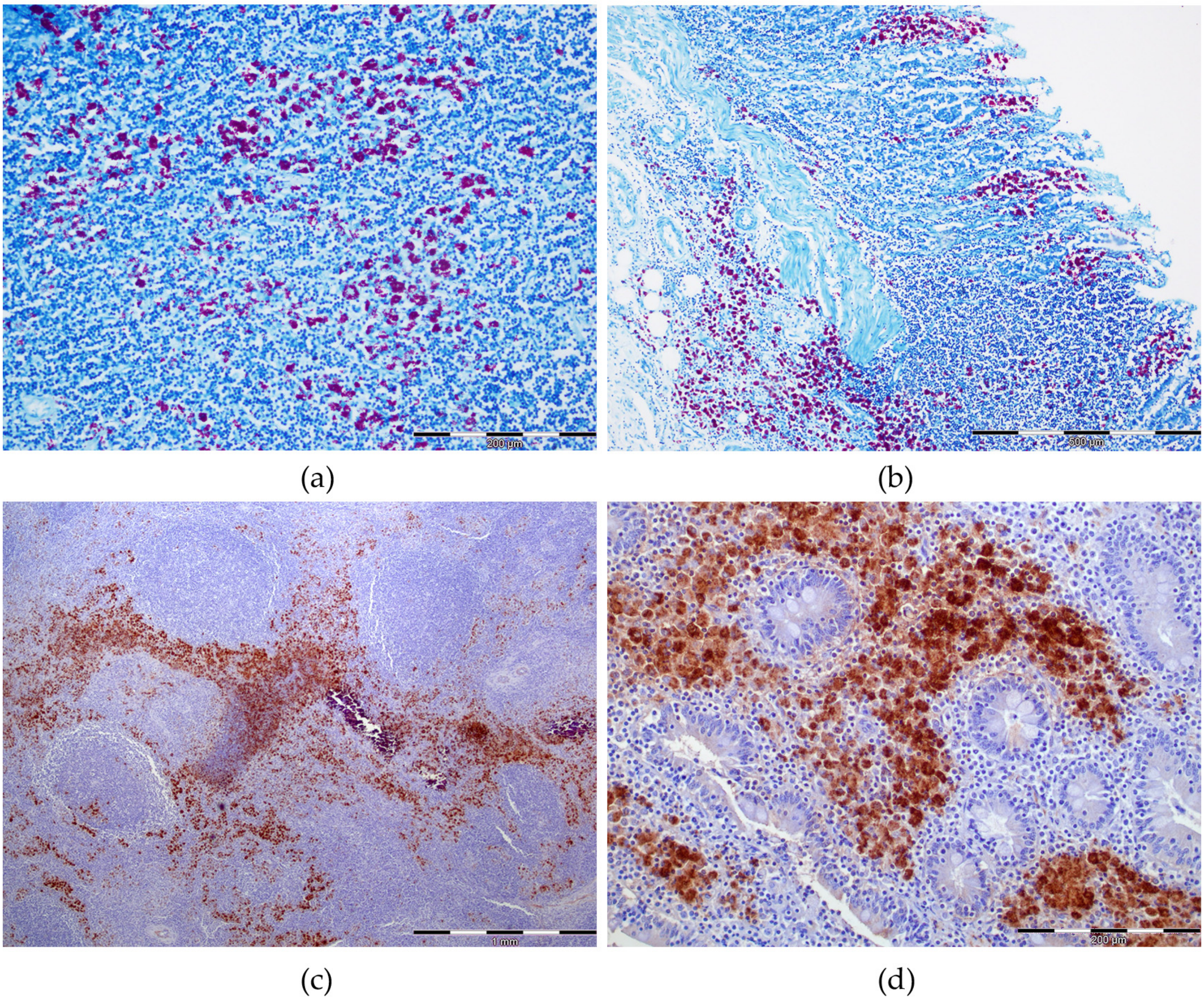


Figure 4. MAP confirmation by Ziehl–Neelsen stain (ZN) and immunohistochemistry (IHC) in mesenteric lymph nodes (MS LNs) and ileocecal valve (ICV). (**a**) MS LN, ZN (scale: 200 µm): numerous acid-fast bacilli (red) in the cytoplasm of macrophages and multinucleated giant cells; (**b**) ICV, ZN (scale: 500 µm): large number of acid-fast bacilli (red) in both lamina propria (LP) and Peyer's patches (PPs) of severely affected ICV; (**c**) LN, IHC (scale: 1 mm): strong immunoreaction in an affected LN showed as brown granular cytoplasmatic stain in macrophages and multinucleated giant cells; (**d**) ICV, IHC (scale: 200 µm): strong brown granular immunolabelling in ICV mucosa.

4. Discussion

Paratuberculosis is an important health issue affecting the goat farm industry worldwide [4]. Complete postmortem analysis is crucial for adequate diagnosis and herd status confirmation [5,6,16,29]. Nevertheless, gross PTB-compatible lesions were only observed in 62% of the animals enrolled in this study and the majority were confined to the MS LNs. Similar results have been previously reported in both experimentally [18] and naturally [16] infected goats, highlighting the importance of the histopathological examination of the MAP target organs.

Regarding the vaccination status, vaccinated animals exhibited gross PTB lesions primarily between 12 and 24 months in contrast with non-vaccinated goats where granulomatous MS LNs lesions were detected macroscopically in all age groups including older animals. Our results agree with previous studies which confirm that the anti-MAP vaccination therapeutic effect consists in the reduction of the severity of lesions and clinical signs in both early vaccinated animals (around 5 months of age) and adult goats [2,10,21,31,32].

Granuloma formation is commonly reported but not always present in MS LNs of goats with PTB in both experimental [18] and natural infection [4,6,16,17]. Thus, an adapted score, proposed by Wangoo et al., 2005 [30], for tuberculosis-induced lesions was applied in the present study. Different scores for PTB histological evaluation in goats have been described, although none of them was selected since they are majorly centered in the PPs and LP lesions and do not grade the MS LNs granuloma stages separately [16,18].

Lesions observed in the MS LNs in our study were characterized by the presence of well-formed granulomas with vast areas of calcification in 24/39 cases, but in only 16/24 were both LAM and GLA described. To our knowledge, there is no data regarding the relation between gross LMA and GLA lesions [4,6,16,17]. Thus, the correct gross inspection of the cut surface is of crucial importance for the macroscopic diagnosis of PTB lesions, even in not apparently enlarged LNs.

MS LNs presented histologically detectable granulomatous lesions in 36/39 of the cases. In 27/36 of the animals, grade IV lesions were described, although in 16/36, grade I microgranulomas were the most frequent lesion type. Those results suggest that granuloma formation has a chronic evolution which has been confirmed in other mycobacterial infections such as cow tuberculosis [30,33,34]. Our findings also highlight that histological confirmation is needed for lesion detection in the early stage of granuloma formation.

Gross PTB lesions affecting the intestines were detected in only 7/39 of the cases. The absence of macroscopic lesions has been previously described as a common finding in goat PTB [3,4,16,17] in contrast with bovine PTB, where classic diffusely thickened corrugated intestinal mucosa folds are commonly observed [6,35]. Some authors describe ulceration of the intestinal mucosa in both clinical [36] and subclinical cases [18], but such findings were not observed in the animals studied. Histological evaluation of the intestine was carried out following the score proposed per Krüger et al., 2015 [18]. This score is based on histological lesions observed only with hematoxylin-eosin stain and is not influenced by AFB load detected with ZN as the one proposed by Corpa et al., 2000 [16], for naturally infected goats [16]. Modifications were introduced to separately evaluate the LP and the PP based on differences described in previous studies regarding the difference between the type of lesions described in those two locations [16,18]. Regarding the histological lesions described, our result agrees with previous studies which suggest that goats have limited ability to control mycobacterial infections and focal lesions are rarely seen [16,37].

MAP confirmation in formalin fixed-paraffin embedded tissues was carried out by ZN and IHC. Moderate correlation was observed between the detection capacity of the two techniques, but the intensity of the reaction was stronger in the case of IHC, agreeing with previous reports [38,39]. Low ZN staining could be explained by the fact that only the intact bacilli take up the stain and IHC anti-MAP antigens can detect dead bacterial cells with compromised cellular wall [38,39].

Etiological confirmation was also performed by qPCR/bacterial culture in 29/39 animals, 13 of which tested positive. Although bacterial culture is considered the gold standard for MAP detection, three animals which tested negative were positive by qPCR, IHC and ZN. Various studies describe qPCR as a more sensitive test for MAP detection in different species than bacterial culture, although data in goats is limited [35,40].

Both PTB and respiratory disease affect the goat farming industry worldwide, causing significant financial losses [12,17,30–33]. Nevertheless, data about the prevalence of both entities in goat herds is limited. In this study respiratory lesions were observed in 64.1% of the animals. Inflammatory lung disease was histologically detected more frequently in animals with PTB lesions in the ICV. Therefore, in naturally infected herds, there could be significant relations between the development of those two processes. Based on PTB pathogenesis and the demonstrated long subclinical stage and chronic onset [6,16,32,41], we could hypothesize that it has been the predisposal factor for lung inflammation in the present study. Additionally, vaccination against *Pasteurella* spp. was commonly issued as a control strategy in the farms studied and could have played a role, although, it is worth mentioning that lung disease in goats is multifactorial, including different causative agents and various environmental, management, herd and breed factors [24,25,42].

Pneumonia lesions had a 17.5% higher prevalence in the non-vaccinated animals in contrast with vaccinated ones. Furthermore, the majority of the vaccinated animals which exhibited histological lesions of pulmonary inflammation had between 12 and 24 months. In non-vaccinated animals, respiratory lesions were diagnosed histologically in different age groups including older animal. Additionally, significantly more non-vaccinated goats presented PTB lesions affecting the ICV, although no such difference was demonstrated regarding the MS LNs. Various studies have confirmed that vaccination against PTB has a generalized beneficial effect, reducing the overall number of losses in the herd, regardless of the cause, although there are no specific reports on reduction of respiratory inflammation [2,10,32,43].

Bronchopneumonia lesions could be associated with opportunistic bacterial pathogens including *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, hemolytic *Streptococci*, *Helcococcus ovis* and *Mycoplasma* spp. [15–17,30]. Interstitial and bronchointerstitial pneumonia, on the other hand, have been described in a broad range of both infectious and non-infectious processes such as virus, mycoplasmosis, parasitosis, protozoal infection, toxicity and hypersensitivity reactions [19,24,25,42,44]. Since histological appearance is similar irrespective to etiology, diagnosis is usually based on clinical signs observed and lesions in other organs [15–17,30], and ancillary tests (e.g., PCR, microbial culture) are needed to identify the aetiologic agent [19,24,25,42,44].

Besides PTB, other lesions were described affecting the gastrointestinal tract including mainly lymphohistiocytic and eosinophilic enteritis which could be associated with parasitic agents such as *Ostertagia*, *Nematodirus* and *Trichostrongylus* [6,19,45].

In the present study, 15.3% more of the non-vaccinated animals presented non-PTB gastrointestinal lesions, although no statistical differences were demonstrated, probably due to the small sample size. Previous studies report a general reduction of the losses in vaccinated herds but do not analyze the different systems separately which were less affected [2,10,32,43]. Our results do suggest that anti-MAP vaccination could in fact have a protective effect against concomitant gastrointestinal pathologies.

Non-PTB lymphadenitis, on the other hand, was detected grossly in more than 50% of the animals. This reflects the invasion of infectious agents in the lymph node resulting from the drainage of products of a distant inflammation and their respective progression involving the node directly [26]. In the cases of *Corynebacterium pseudotuberculosis* compatible CLA, where LNs are the target organs, our results reported RPh, MD, and MA LNs as common lesion sites, which agrees with recent reports in goats [46,47].

The liver was mainly affected by randomly distributed multifocal lymphohistiocytic hepatitis. This unspecific inflammatory pattern is frequently reported in ruminants as secondary to systemic bacteremia caused by the agents affecting other organs [6,27]. Hepatocellular steatosis was also observed both grossly and histologically. Since all animals included presented PTB-attributed weight loss, the steatosis is most likely caused by the excessive entry of fatty acids into the liver due to their increased metabolization from the adipose tissue [6,27]. The granulomatous hepatitis recorded in only three animals could be associated with severe cases of PTB due to MAP spreading [4,6].

The cardiovascular system presented gross lesions only affecting the pericardium. The main type detected was pericarditis, which was both macroscopically and microscopically confirmed. Pericarditis is commonly associated with hematogenous microbial infections, with a high prevalence of association with pneumonic lesions [24,25,48], which were detected in the animals analyzed. Non-suppurative myocarditis was described histologically in animals and could be associated with a wide variety of systemic diseases but is rarely primary [48]. The *Sarcocystis* spp. cysts reported histologically in the studied animals are a very common finding in ruminants and an increase in the numbers of cysts with age is confirmed [48].

Regarding the urinary system, interstitial nephritis was the most common histological finding, which was rarely detected grossly, which agrees with results of previous studies in goats [19,49].

5. Conclusions

The present study describes the most common concomitant pathologies found in goat herds with natural PTB infection mainly affecting the respiratory and gastrointestinal tracts. Our results emphasize the importance of routine histological examination for disease assessment, as well as the importance of anatomic pathology as a useful tool for the diagnosis and management of PTB in goat herds, since the majority of the described findings were only detectable histologically. Furthermore, vaccination against PTB might potentially have a positive effect on the reduction of respiratory and gastrointestinal pathologies, based on the findings described and the statistically significant differences demonstrated between the vaccinated and non-vaccinated animals. Further studies on a greater scale are needed to determine and understand the multiple etiologies and factors associated with the pathologies described, as well as the effect of PTB vaccination on their prevalence.

Author Contributions: Conceptualization, Y.P.-S., Ó.Q.-C., V.P., A.C. and M.A.; methodology, E.P.S., Y.P.-S., Ó.Q.-C., V.P. and M.A.; validation, E.P.S., Y.P.-S., Ó.Q.-C., V.P. and M.A.; formal analysis, Y.P.-S., Ó.Q.-C. and M.A.; investigation, E.P.S., Y.P.-S., Ó.Q.-C., V.P., A.C. and M.A.; resources, A.E.d.l.M., M.A.R., Ó.Q.-C. and M.A.; data curation, M.d.P.Q.-M. and M.A.; writing—original draft preparation, E.P.S., Y.P.-S., Ó.Q.-C., M.J.C. and M.A.; writing—review and editing, E.P.S., Y.P.-S., Ó.Q.-C., M.d.P.Q.-M., M.J.C. and M.A.; visualization, E.P.S., Y.P.-S., Ó.Q.-C. and M.A.; supervision, A.E.d.l.M., M.A.R., M.J.C. and M.A.; project administration, E.P.S., Ó.Q.-C., Y.P.-S., A.E.d.l.M., M.A.R. and M.A.; funding acquisition, A.E.d.l.M., M.A.R. and M.A. All authors have read and agreed to the published version of the manuscript.

Funding: 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. Elena Plamenova Stefanova was the recipient of a predoctoral contract of the The Ministry of Universities, Spain (FPU20/03693) and Charter100 2021 scholarship for PhD students' mobility.

Institutional Review Board Statement: This manuscript has been exempted by the Ethical Committee for Animal Experimentation of the University of Las Palmas of Gran Canaria (OEBA-ULPGC-13/2023), which notified that the procedure falls within the exceptions; thus, 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 Ethical Committee for Animal Experimentation.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

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 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 technician Marta Silva Samos for the knowledge exchange and laboratory training, and the VISAVET Health Surveillance, Complutense University of Madrid, Madrid, Spain for performing the bacterial culture.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Summary of pathological findings in 39 naturally infected goats with PTB lesions.

System	Organ	Gross	Frequency (%)	Histology	Frequency (%)
Hemolymphatic	MS	LAM GLA	21 (53.8%) 24 (61.5%)	GLA	36 (92.3%)
	RPh	GLA CLA Abscess	2 (5.1%) 2 (5.1%) 3 (7.7%)	SLA/CLA	8 (20.5%)
	PE	SLA Abscess	6 (15.4%) 4 (10.3%)	SLA/CLA	4 (10.3%)
	MD	SLA CLA Abscess	3 (7.7%) 3 (7.7%) 2 (5.1%)	SLA/CLA	8 (20.5%)
	MA	SLA GLA CLA Abscess	1 (2.6%) 2 (5.1%) 2 (5.1%) 1 (2.6%)	SLA/CLA	4 (10.3%)
Respiratory	Lungs	CBP CBP + fibrinous pleuritis CBP + fibrous pleuritis	4 (10.3%) 7 (17.9%) 5 (12.8%)	FPBP/FNBP IP BIP Pleuritis only	12 (30.8%) 6 (15.4%) 3 (7.7%) 4 (10.3%)
Gastrointestinal	Intestine	Diffusely thickened appearance	10 (25.6%)	EE LPE	2 (5.1%) 1 (2.6%)
	Abomasum	PTB-compatible enteritis Hemorrhagic enteritis	7 (17.9%) 1 (2.6%)	GE Abomasitis/abomasal ulcer	32(82.1%) 4 (10.3%)
	Liver	Steatosis Capsular fibrosis Mineralizations	7 (17.9%) 6 (15.4%) 1 (2.6%)	Steatosis Lymphohistiocytic hepatitis Granulomatous hepatitis	11 (28.2%) 25 (64.1%) 3 (7.7%)
Urinary	Kidney	Interstitial nephritis	2 (5.1%)	Interstitial nephritis	10 (25.6%)
Cardiovascular	Heart	Hydropericardium Fibrous pericarditis Petechial hemorrhages	2 (5.1%) 2 (5.1%) 2 (5.1%)	Non-suppurative myocarditis Fibrous pericarditis Myocardial sarcocystosis	3 (7.7%) 3 (7.7%) 9 (23.1%)

MS, mesenteric lymph nodes; RPh, retropharyngeal lymph nodes; PE, prescapular lymph nodes; MD, mediastinal lymph nodes; MA, mammary lymph nodes; LAM, lymphadenomegaly; GLA, granulomatous lymphadenitis; SLA, suppurative lymphadenitis; CLA, caseous lymphadenitis; CBP, cranioventral bronchopneumonia; FNBP, fibrinonecrotic brochopneumonia; FPBP, fibrinopurulent bronchopneumonia; IP, interstitial pneumonia; BIP, bronchointerstitial pneumonia; GE, granulomatous enteritis; EE, eosinophilic enteritis; LPE, lymphoplasmacytic enteritis.

References

1. Karuppusamy, S.; Kirby, G.M.; Mutharia, L.; Tripathi, B.N. An Update on Mycobacterium Avium Subspecies Paratuberculosis Antigens and Their Role in the Diagnosis of Johne's Disease. *World J. Microbiol. Biotechnol.* **2019**, *35*, 120. [CrossRef] [PubMed]
2. Fernández, M.; Royo, M.; Fuertes, M.; Arteche-Villasol, N.; Ferreras, M.C.; Benavides, J.; Pérez, V. Effects of Paratuberculosis Vaccination at Different Ages in a Dairy Goat Herd: A 2-Year Follow-Up. *Animals* **2022**, *12*, 3135. [CrossRef] [PubMed]
3. Paliwal, O.P.; Rehbinder, C. Ultrastructural studies of paratuberculosis (Johne's disease) in goats. *Acta Vet. Scand.* **1981**, *22*, 180. [CrossRef] [PubMed]

4. Idris, S.M.; Eltom, K.H.; Okuni, J.B.; Ojok, L.; Elmagzoub, W.A.; El Wahed, A.A.; Eltayeb, E.; Gameel, A.A. Paratuberculosis: The Hidden Killer of Small Ruminants. *Animals* **2022**, *12*, 12. [CrossRef] [PubMed]
5. Benavides, J.; González, L.; Dagleish, M.; Pérez, V. Diagnostic Pathology in Microbial Diseases of Sheep or Goats. *Vet. Microbiol.* **2015**, *181*, 15–26. [CrossRef]
6. Uzal, F.A.; Plattner, B.L.; Hostetter, J.M. Chapter 1—Alimentary System. In *Jubb, Kennedy & Palmer's Pathology of Domestic Animals*, 6th ed.; Maxie, M.G., Ed.; W.B. Saunders: Philadelphia, PA, USA, 2016; Volume 2, pp. 1–257.e2. ISBN 978-0-7020-5318-4.
7. Garcia, A.; Shalloo, L. Invited Review: The Economic Impact and Control of Paratuberculosis in Cattle. *J. Dairy Sci.* **2015**, *98*, 5019–5039. [CrossRef]
8. Barrero-Domínguez, B.; Luque, I.; Huerta, B.; Gomez-Laguna, J.; Galán-Relaño, Á.; Gómez-Gascón, L.; Sánchez, M.; Astorga, R.J. Paratuberculosis in Dairy Goat Flocks from Southern Spain: Risk Factors Associated with Seroprevalence; Paratuberculosis in Dairy Goat Flocks from Southern Spain: Risk Factors Associated with Seroprevalence. *Vet. Rec.* **2019**, *185*, 600. [CrossRef]
9. Delgado, L.; Marín, J.F.G.; Muñ Oz, M.; Benavides, J.; Juste, R.A.; García-Pariente, C.; Fuertes, M.; González, J.; Ferreras, M.C.; Pé Rez, V. Infectious Disease Research Pathological Findings in Young and Adult Sheep Following Experimental Infection with 2 Different Doses of Mycobacterium Avium Subspecies Paratuberculosis. *Vet. Pathol.* **2013**, *50*, 857–866. [CrossRef]
10. Agulló-Ros, I.; Andrada, M.; Pérez-Sancho, M.; Roy, Á.; Bezos, J.; Bonnet, T.; Moreno, I.; Paz-Sánchez, Y.; Domínguez, M.; Gómez-Villamandos, J.C.; et al. Effect of Heat-Inactivated Mycobacterium Avium Subspecies Paratuberculosis (MAP) Vaccine on the Lesions and Immunopathology Developed in Target Tissues of Naturally MAP-Infected Goats. *Vet. Microbiol.* **2022**, *273*, 109543. [CrossRef]
11. Stau, A.; Seelig, B.; Walter, D.; Schroeder, C.; Ganter, M. Seroprevalence of Mycobacterium Avium Subsp. Paratuberculosis in Small Ruminants in Germany. *Small Rumin. Res.* **2012**, *105*, 361–365. [CrossRef]
12. Mercier, P.; Baudry, C.; Beaudeau, F.; Seegers, H.; Malher, X. Estimated Prevalence of Mycobacterium Avium Subspecies Paratuberculosis Infection in Herds of Dairy Goats in France. *Vet. Rec.* **2010**, *167*, 412–415. [CrossRef] [PubMed]
13. Hosseiniporgham, S.; Cubeddu, T.; Rocca, S.; Sechi, L.A. Identification of Mycobacterium Avium Subsp. Paratuberculosis (Map) in Sheep Milk, a Zoonotic Problem. *Microorganisms* **2020**, *8*, 1264. [CrossRef]
14. Hosseiniporgham, S.; Rebechesu, L.; Pintore, P.; Lollai, S.; Dattena, M.; Russo, S.; Ruiu, A.; Sechi, L.A. A Rapid Phage Assay for Detection of Viable Mycobacterium Avium Subsp. Paratuberculosis in Milk. *Sci. Rep.* **2022**, *12*, 475. [CrossRef]
15. De Juan Ferré, L.; Domínguez Rodríguez, L.; Suárez Fernández, G.; Mateos García, A. *Paratuberculosis Caprina: Aportaciones a Su Diagnóstico, Epidemilogía Molecular y Control*; Universidad Complutense de Madrid: Madrid, Spain, 2005; ISBN 8466928529.
16. Corpa, J.M.; Garrido, J.; García Marín, J.F.; Pérez, V. Classification of Lesions Observed in Natural Cases of Paratuberculosis in Goats. *J. Comp. Pathol.* **2000**, *122*, 255–265. [CrossRef]
17. Tafti, A.K.; Rashidi, K. The Pathology of Goat Paratuberculosis: Gross and Histopathological Lesions in the Intestines and Mesenteric Lymph Nodes. *J. Vet. Med. B* **2000**, *47*, 487–495. [CrossRef] [PubMed]
18. Krüger, C.; Köhler, H.; Liebler-Tenorio, E.M. Sequential Development of Lesions 3, 6, 9, and 12 Months After Experimental Infection of Goat Kids with Mycobacterium Avium Subsp Paratuberculosis. *Vet. Pathol.* **2015**, *52*, 276–290. [CrossRef] [PubMed]
19. Smith, M.C.; Sherman, D.M. *Goat Medicine*; John Wiley & Sons: Hoboken, NJ, USA, 2009; ISBN 0781796431.
20. Derakhshandeh, A.; Namazi, F.; Khatamsaz, E.; Eraghi, V.; Hemati, Z. Goat Paratuberculosis in Shiraz: Histopathological and Molecular Approaches. *Vet. Res. Forum* **2018**, *9*, 253–257. [CrossRef]
21. Bastida, F.; Juste, R.A. Paratuberculosis Control: A Review with a Focus on Vaccination. *J. Immune Based Ther. Vaccines* **2011**, *9*, 8–17. [CrossRef]
22. Van Metre, D.C.; Tyler, J.W.; Stehman, S.M. Diagnosis of enteric disease in small ruminants. *Vet. Clin. N. Am. Food Anim. Pract.* **2000**, *16*, 87–115. [CrossRef]
23. Tariq, K.A. A Review of the Epidemiology and Control of Gastrointestinal Nematode Infections of Small Ruminants. *Proc. Natl. Acad. Sci. India Sect. B-Biol. Sci.* **2015**, *85*, 693–703. [CrossRef]
24. Caswell, J.L.; Williams, K.J. Chapter 5—Respiratory System. In *Jubb, Kennedy & Palmer's Pathology of Domestic Animals*, 6th ed.; Maxie, M.G., Ed.; W.B. Saunders: Philadelphia, PA, USA, 2016; Volume 2, pp. 465–591.e4. ISBN 978-0-7020-5318-4.
25. Chakraborty, S.; Kumar, A.; Tiwari, R.; Rahal, A.; Malik, Y.; Dhama, K.; Pal, A.; Prasad, M. Review Article Advances in Diagnosis of Respiratory Diseases of Small Ruminants. *Veter-Med. Int.* **2014**, *2014*, 1–16. [CrossRef] [PubMed]
26. Valli, V.E.O.; Kiupel, M.; Bienzle, D.; Wood, R.D. Chapter 2—Hematopoietic System. In *Jubb, Kennedy & Palmer's Pathology of Domestic Animals*, 6th ed.; Maxie, M.G., Ed.; W.B. Saunders: Philadelphia, PA, USA, 2016; Volume 3, pp. 102–268.e1. ISBN 978-0-7020-5319-1.
27. Brown, D.L.; Van Wettere, A.J.; Cullen, J.M. Chapter 8—Hepatobiliary System and Exocrine Pancreas1. In *Pathologic Basis of Veterinary Disease*, 6th ed.; Zachary, J.F., Ed.; Mosby: London, UK, 2017; pp. 412–470.e1. ISBN 978-0-323-35775-3.
28. Cullen, J.M.; Stalker, M.J. Chapter 2—Liver and Biliary System. In *Jubb, Kennedy & Palmer's Pathology of Domestic Animals*, 6th ed.; Maxie, M.G., Ed.; W.B. Saunders: Philadelphia, PA, USA, 2016; Volume 2, pp. 258–352.e1. ISBN 978-0-7020-5318-4.
29. King, J.M.; Roth-Johnson, L.; Dodd, D.C.; Newsom, M.E. *The Necropsy Book: A Guide for Veterinary Students, Residents, Clinicians, Pathologists, and Biological Researchers*; The Internet-First University Press: Ithaca, NY, USA, 2014.
30. Wangoo, A.; Johnson, L.; Gough, J.; Ackbar, R.; Inglut, S.; Hicks, D.; Spencer, Y.; Hewinson, G.; Vordermeier, M. Advanced Granulomatous Lesions in Mycobacterium Bovis-Infected Cattle Are Associated with Increased Expression of Type I Procollagen, Γδ (WC1+) T Cells and CD 68+ Cells. *J. Comp. Pathol.* **2005**, *133*, 223–234. [CrossRef]

31. Juste, R.A.; Perez, V. Control of Paratuberculosis in Sheep and Goats. *Vet. Clin. N. Am. Food Anim. Pract.* **2011**, *27*, 127–138. [CrossRef]
32. Corpa, J.M.; Pérez, V.; García Marín, J.F.; Sánchez, M.A. Control of Paratuberculosis (Johne's Disease) in Goats by Vaccination of Adult Animals. *Vet. Rec.* **2000**, *146*, 195–196. [CrossRef] [PubMed]
33. Domingo, M.; Vidal, E.; Marco, A. Pathology of Bovine Tuberculosis. *Res. Vet. Sci.* **2014**, *97*, S20–S29. [CrossRef] [PubMed]
34. Cassidy, J.P.; Bryson, D.G.; Pollock, J.M.; Evans, R.T.; Forster, F.; Neill, S.D. Early Lesion Formation in Cattle Experimentally Infected with Mycobacterium Hovis. *J. Comp. Pathol.* **1998**, *119*, 27–44. [CrossRef]
35. Gilardoni, L.R.; Paolicchi, F.A.; Mundo, S.L. Diagnosis of Bovine Paratuberculosis 201 Bovine Paratuberculosis: A Review of the Advantages and Disadvantages of Different Diagnostic Tests. *Rev. Argent. Microbiol.* **2012**, *44*, 201–215. [PubMed]
36. Lybeck, K.R.; Storset, A.K.; Djønne, B.; Valheim, M.; Olsen, I. Faecal Shedding Detected Earlier than Immune Responses in Goats Naturally Infected with Mycobacterium Avium Subsp. Paratuberculosis. *Res. Vet. Sci.* **2011**, *91*, 32–39. [CrossRef]
37. Gutiérrez, M.; Samper, S.; Gavigan, J.; Francisco Garci, J.; Mari, A.; Marti, C. Differentiation by Molecular Typing of Mycobacterium Bovis Strains Causing Tuberculosis in Cattle and Goats. *J. Clin. Microbiol.* **1995**, *33*, 2953–2956. [CrossRef]
38. Thoresen, O.F.; Falk, K.; Evensen, Ø. Comparison of Immunohistochemistry, Acid-Fast Staining, and Cultivation for Detection of Mycobacterium Paratuberculosis in Goats. *J. Vet. Diagn. Investig.* **1994**, *6*, 195–199. [CrossRef]
39. Kheirandish, R.; Tafti, A.K.; Hosseini, A. Classification of Lesions and Comparison of Immunohistochemical and Acid Fast Staining in Diagnosis of Naturally Occurring Paratuberculosis in Goats. *Small Rumin. Res.* **2009**, *87*, 81–85. [CrossRef]
40. Bögli-Stuber, K.; Kohler, C.; Seitert, G.; Glanemann, B.; Antognoli, M.C.; Salman, M.D.; Wittenbrink, M.M.; Wittwer, M.; Wassenaar, T.; Jemmi, T.; et al. Detection of Mycobacterium Avium Subspecies Paratuberculosis in Swiss Dairy Cattle by Real-Time PCR and Culture: A Comparison of the Two Assays. *J. Appl. Microbiol.* **2005**, *99*, 587–597. [CrossRef] [PubMed]
41. Sweeney, R.W. Pathogenesis of Paratuberculosis. *Vet. Clin. N. Am. Food Anim. Pract.* **2011**, *27*, 537–546. [CrossRef] [PubMed]
42. Matthews, J.G. Chapter 15—Respiratory System. In *Diseases of the Goat*; John Wiley & Sons: Hoboken, NJ, USA, 2016; pp. 258–274. ISBN 9781119073543.
43. Mercier, P.; Brémaud, I.; Gautier, M.P. Vaccination of Kids under One Month of Age with a Killed Vaccine and Reduction in the Frequency of Faecal Shedding of Mycobacterium Avium Subspecies Paratuberculosis. *Small Rumin. Res.* **2014**, *121*, 425–433. [CrossRef]
44. Carvallo, F.R.; Stevenson, V.B. Interstitial Pneumonia and Diffuse Alveolar Damage in Domestic Animals. *Vet. Pathol.* **2022**, *59*, 586–601. [CrossRef]
45. Matthews, J.G. Chapter 14—Diarrhoea. In *Diseases of the Goat*; John Wiley & Sons: Hoboken, NJ, USA, 2016; pp. 204–231. ISBN 9781119073543.
46. Debien, E.; Hélie, P.; Buczinski, S.; Leboeuf, A.; Bélanger, D.; Drolet, R. Article Proportional Mortality: A Study of 152 Goats Submitted for Necropsy from 13 Goat Herds in Quebec, with a Special Focus on Caseous Lymphadenitis. *Can. Vet. J.* **2013**, *54*, 581.
47. Pointon, A.; Hamilton, D.; Kiermeier, A. Comparison of Postmortem Inspection Procedures for Detecting Caseous Lymphadenitis of Australian Sheep and Goats. *Vet. Rec.* **2019**, *185*, 54. [CrossRef]
48. Robinson, W.F.; Robinson, N.A. Chapter 1—Cardiovascular System. In *Jubb, Kennedy & Palmer's Pathology of Domestic Animals*, 6th ed.; Maxie, M.G., Ed.; W.B. Saunders: Philadelphia, PA, USA, 2016; Volume 3, pp. 1–101.e1. ISBN 978-0-7020-5319-1.
49. Cianciolo, R.E.; Mohr, F.C. Chapter 4—Urinary System. In *Jubb, Kennedy & Palmer's Pathology of Domestic Animals*, 6th ed.; Maxie, M.G., Ed.; W.B. Saunders: Philadelphia, PA, USA, 2016; Volume 2, pp. 376–464.e1. ISBN 978-0-7020-5318-4.

CAPÍTULO II

Detección de la paratuberculosis caprina (enfermedad de Johne) en rebaños antes y después de la vacunación: diagnóstico morfológico, clasificación de lesiones e identificación bacteriana

6. CAPÍTULO II

6.1. RESUMEN

Detección de la paratuberculosis caprina (enfermedad de Johne) en rebaños antes y después de la vacunación: diagnóstico morfológico, clasificación de lesiones e identificación bacteriana

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

La paratuberculosis (PTB), también conocida como enfermedad de Johne, es una enfermedad infecciosa crónica de declaración obligatoria con distribución mundial afectando a rumiantes domésticos y salvajes. Es causada por *Mycobacterium avium* subespecie *paratuberculosis* (MAP) y cursa con enteritis granulomatosa, siendo la válvula iliocecal (VIC) importante para el correcto diagnóstico, y con linfoadenitis granulomatosa de los linfonodos mesentéricos (LN MS). El diagnóstico temprano es complejo, ya que los signos clínicos suelen aparecer en fases avanzadas, por lo que las pruebas de laboratorio y la observación de lesiones histológicas son fundamentales.

En el presente estudio realizado en una granja con un historial confirmado de PTB y libre de tuberculosis, se recogieron muestras en matadero de LN MS y VIC de 105 cabras sin signos clínicos de PTB, de las cuales 61 no estaban vacunadas y 44 habían sido vacunadas contra MAP. Se evaluaron las lesiones granulomatosas compatibles con PTB en LN MS clasificándolos desde 0, sin lesiones, hasta grado IV, necrosis y mineralización. En la VIC, la lámina propia (LP) de la VIC y las placas de Peyer (PP) se evaluó, de manera separada en términos de severidad y distribución, el infiltrado granulomatoso. Además, se cuantificó la carga de bacilos ácido-alcohol resistentes mediante la tinción de Ziehl-Neelsen (ZN), antígenos de MAP mediante inmunohistoquímica (IHQ), y el ADN de MAP por PCR dirigida contra la secuencia IS900.

El 39% de las cabras mostró lesiones macroscópicas compatibles con PTB, con una prevalencia del 31,72% en las vacunadas y del 68,29% en las no vacunadas. Las lesiones histopatológicas inducidas por MAP se observaron en el 58,10% de los animales, distribuidas en un 36,07% de las cabras vacunadas y un 63,93% de las no vacunadas (p=0,013). La incorporación de la histopatología como herramienta diagnóstica permitió un aumento del 28%

en los casos diagnosticados en LN MS y del 86,05% en la VIC. Los granulomas de grado IV, caracterizados por mineralización y necrosis central, fueron las lesiones más comunes en los LN MS. En la VIC, predominó una enteritis granulomatosa leve multifocal con macrófagos epitelioides, que se observaron más frecuentemente en las PP que en la LP. Además, se encontraron diferencias estadísticamente significativas en la presencia de lesiones histopatológicas entre las cabras vacunadas y no vacunadas en LN MS ($p=0,018$), LP ($p=0,027$) y PP ($p=0,028$). Los resultados de ZN, IHQ y PCR coincidieron en 63,81% de los casos y los animales no vacunados mostraron mayores tasas de positividad en las tres pruebas con $p<0.05$.

Estos resultados resaltan los beneficios de la vacunación contra MAP en la reducción de lesiones compatibles con PTB y la carga bacteriana en los órganos afectados. Sin embargo, la concordancia entre el ZN y la IHQ fue moderada ($\kappa=0,419$), entre el ZN y la PCR fue baja ($\kappa=0,355$) y entre IHQ y PCR fue moderada ($\kappa=0,444$). Este estudio evidencia la necesidad de integrar análisis macroscópicos e histopatológicos con diversas técnicas de laboratorio para un protocolo de diagnóstico preciso de la PTB subclínica, tanto en cabras vacunadas, como no vacunadas.

frontiers | Frontiers in Veterinary Science

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

Check for updates

OPEN ACCESS

EDITED BY
Cinzia Marianelli,
National Institute of Health (ISS), Italy

REVIEWED BY
Flavia Pruiti Ciarello,
Experimental Zooprophylactic Institute of Sicily (IZSSi), Italy
Monika Hilbe,
University of Zurich, Switzerland

*CORRESPONDENCE
Eva Sierra
eva.sierra@ulpgc.es
Oscar Quesada-Canales
oscar.quesada@ulpgc.es

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

Elena Plamenova Stefanova[1,2], Eva Sierra[1,2]*, Antonio Fernández[1,2], Oscar Quesada-Canales[1,2]*, Yania Paz-Sánchez[1], Ana Colom-Rivero[1], Antonio Espinosa de los Monteros[1,2], Pedro Herráez[1,2], Lucas Domínguez[3,4], Javier Bezos[3,4], Marta Pérez-Sancho[3,4], Inmaculada Moreno[5], María A. Risalde[6,7] and Marisa Andrada[1,2]

[1]Division of Animal Histology and Pathology, Veterinary School, Institute of Animal Health and Food Safety (IUSA), University of Las Palmas de Gran Canaria, Arucas, Spain, [2]Departament of Morphology, Veterinary School, University of Las Palmas de Gran Canaria, Arucas, Spain, [3]VISAVET Health Surveillance Centre, Complutense University of Madrid, Madrid, Spain, [4]Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain, [5]Servicio de Inmunología Microbiana, Centro Nacional de Microbiología, Instituto de Investigación Carlos III, Madrid, Spain, [6]Departamento de Anatomía y Anatomía Patológica Comparadas y Toxicología, Grupo de Investigación GISAZ, UIC Zoonosis y Enfermedades Emergentes ENZOEM, Universidad de Córdoba, Campus de Rabanales, Edificio Sanidad Animal, Córdoba, Spain, [7]Centro de Investigación Biomédica en Red Enfermedades Infecciosas, Instituto de Salud Carlos III (CIBERINFEC, ISCIII), Madrid, Spain

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).

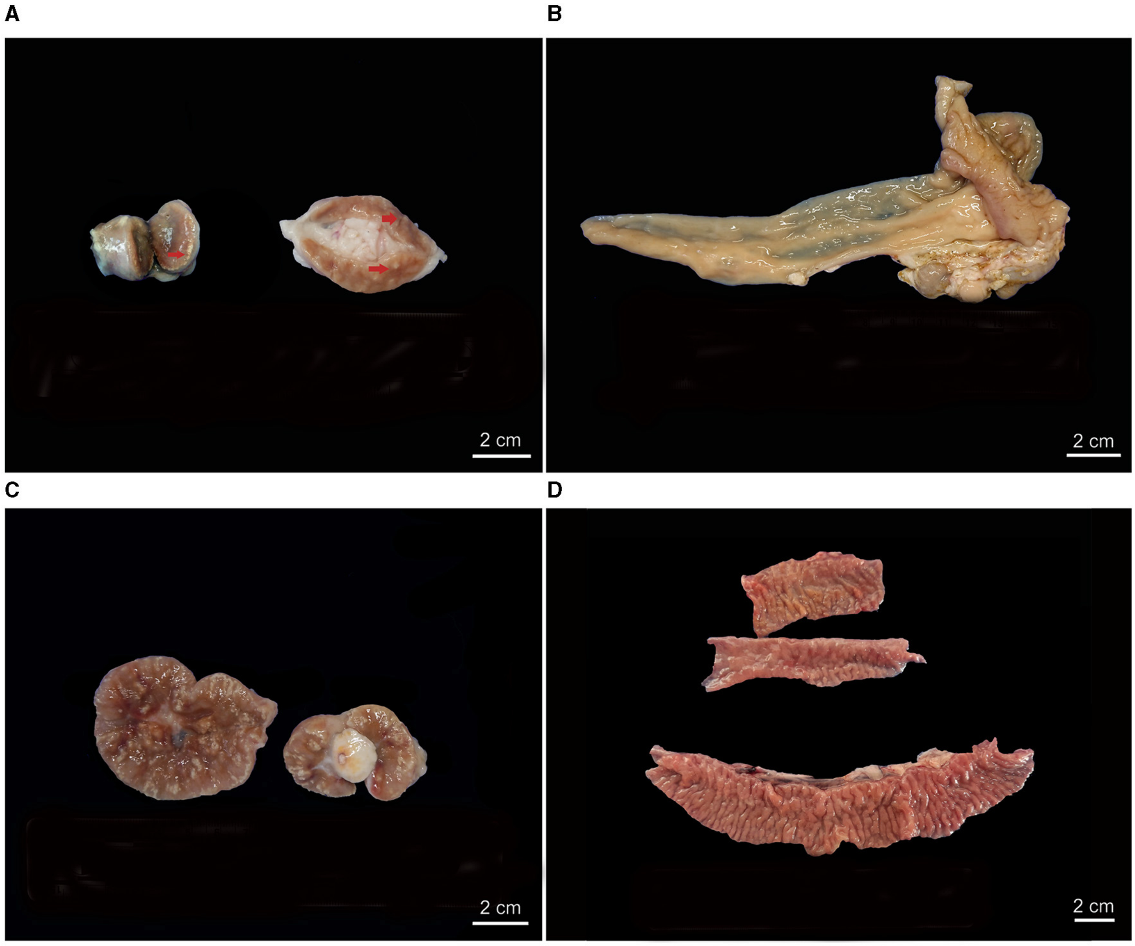


FIGURE 1
Gross PTB-compatible lesions in vaccinated (V) and non-vaccinated (nV) goats. **(A)** Lymphadenomegaly of mesenteric lymph nodes (MS LNs) of a V animal with multifocal white calcified granulomas on the cut surface (arrow). **(B)** Ileocecal valve (ICV) of a V animal with no gross lesions. **(C)** 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

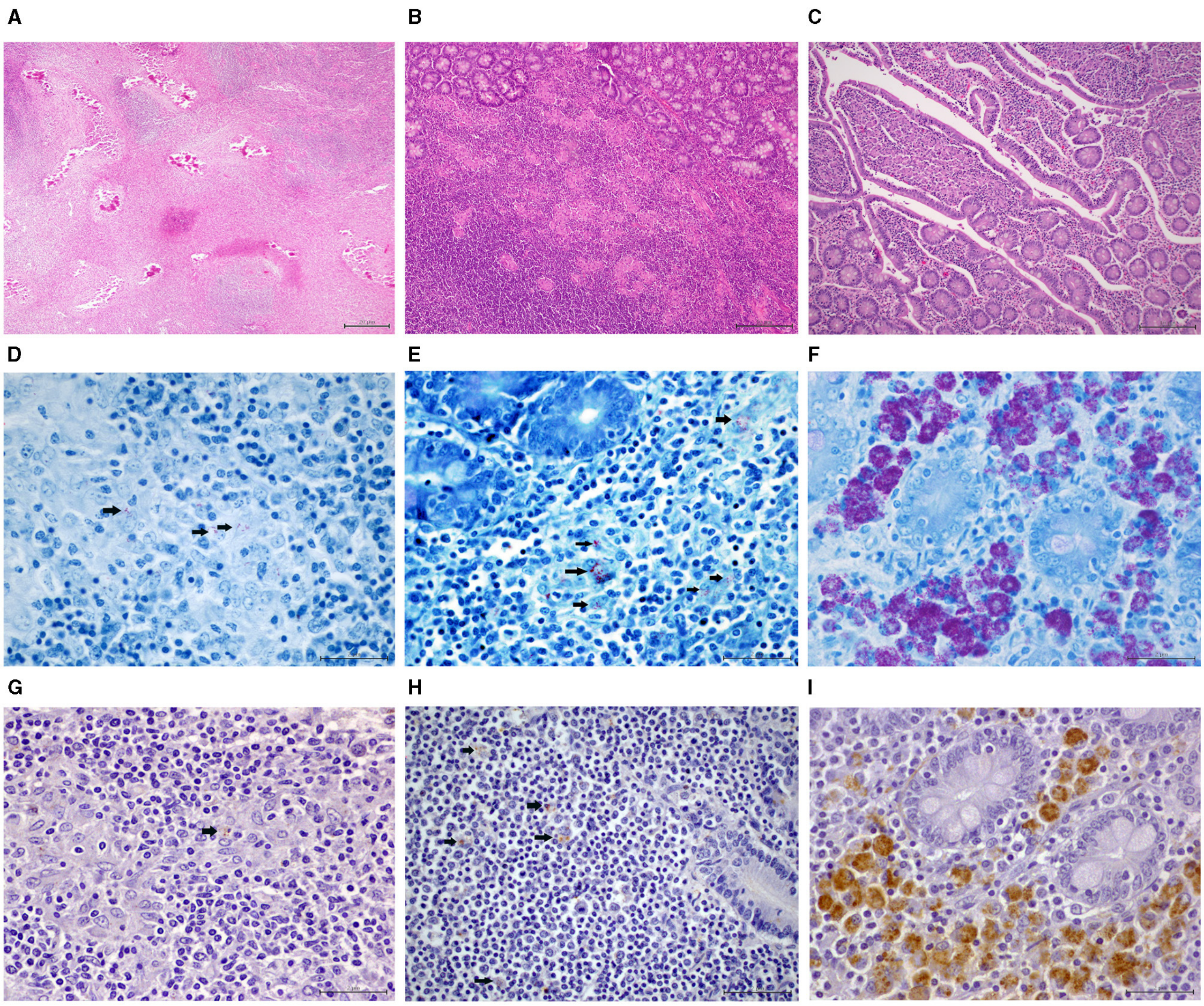


FIGURE 2
Histopathological PTB-compatible findings in vaccinated (V) and non-vaccinated (nV) goats. **(A)** Mesenteric lymph node (MS LN): grade IV lesions with multifocal severe calcified granulomas. HE 4x **(B)** Peyer's patches (PPs) of the ileocecal valve region (ICV): multifocal mild granulomatous 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 (κ = 0.513) in contrast with the results from the ICV samples, where the agreement was low (κ = 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,

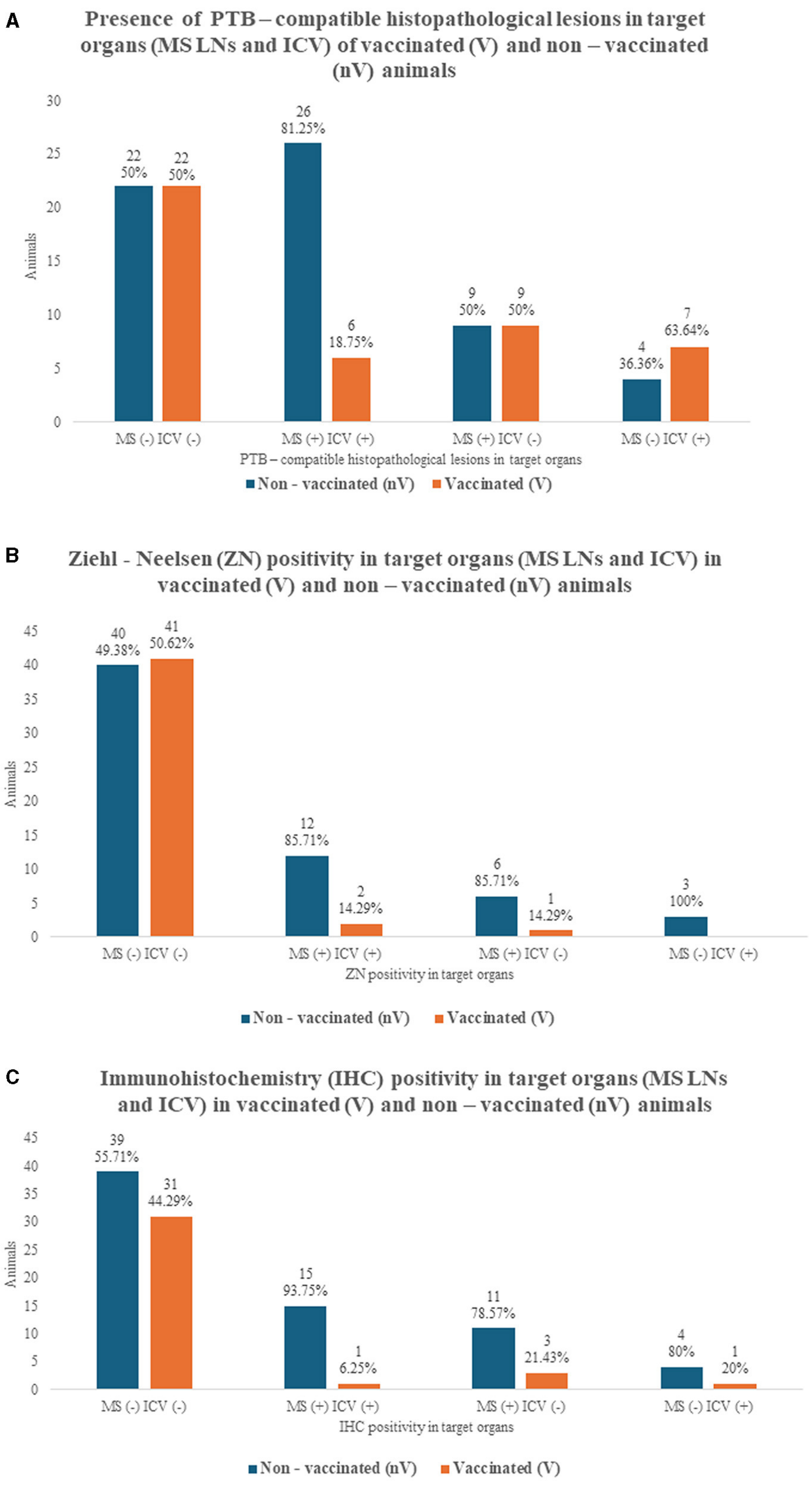


FIGURE 3
Histopathological PTB-compatible lesions, Ziehl-Neelsen (ZN), and immunohistochemistry (IHC) in target organs of 105 naturally infected goats (vaccinated and non-vaccinated). **(A)** PTB-compatible histopathological findings in mesenteric lymph nodes (MS LNs) and/or ileocecal valve region (ICV)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

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Garcia A, Shalloo L. Invited review: the economic impact and control of paratuberculosis in cattle. *J Dairy Sci.* (2015) 98:5019–39. doi: 10.3168/jds.2014-9241

2. Barrero-Domínguez B, Luque I, Huerta B, Gomez-Laguna J, Galán-Relaño Á, Gómez-Gascón L, Sánchez M, Astorga RJ. Paratuberculosis in dairy goat flocks from southern Spain: risk factors associated with seroprevalence. *Vet Record.* (2019) 185:105465. doi: 10.1136/vetrec-2019-105465

3. Delgado L, Marín JFG, Muñóz M, Benavides J, Juste RA, García-Pariente C, et al. Infectious disease research pathological findings in young and adult sheep following experimental infection with 2 different doses of *Mycobacterium avium* subspecies *paratuberculosis*. *Vet Pathol.* (2013) 50:857–66. doi: 10.1177/0300985813476066

4. Karuppusamy S, Kirby GM, Mutharia L, Tripathi BN. An update on *Mycobacterium avium* subspecies *paratuberculosis* antigens and their role in the diagnosis of Johne's disease. *World J Microbiol Biotechnol.* (2019) 35:120. doi: 10.1007/s11274-019-2691-0

5. Idris SM, Eltom KH, Okuni JB, Ojok L, Elmagzoub WA, Wahed AA El, Eltayeb E, Gameel AA. Paratuberculosis: the hidden killer of small ruminants. *Animals.* (2022) 12:12. doi: 10.3390/ani12010012

6. Agulló-Ros I, Andrada M, Pérez-Sancho M, Roy Á, Bezos J, Bonnet T, et al. Effect of heat-inactivated *Mycobacterium avium* subspecies *paratuberculosis* (MAP) vaccine on the lesions and immunopathology developed in target tissues of naturally MAP-infected goats. *Vet Microbiol.* (2022) 273:109543. doi: 10.1016/J.VETMIC.2022.109543

7. De Juan Ferré L, Domínguez Rodríguez L, Suárez Fernández G, Mateos García A. *Paratuberculosis caprina: aportaciones a su diagnóstico, epidemilogía molecular y control*. Madrid: Universidad Complutense de Madrid (2005). 291 p.

8. Espinosa J, Fernández M, Royo M, Grau A, Ángel Collazos J, Benavides J, et al. Influence of vaccination against paratuberculosis on the diagnosis of caprine tuberculosis during official eradication programmes in Castilla y León (Spain). *Transbound Emerg Dis.* (2021) 68:692–703. doi: 10.1111/tbed.13732

9. Arteche-Villasol N, Gutiérrez-Expósito D, Vallejo R, Espinosa J, Elguezabal N, Ladero-Auñon I, Royo M, del Carmen Ferreras M, Benavides J, Pérez V. Early response of monocyte-derived macrophages from vaccinated and non-vaccinated goats against in vitro infection with *Mycobacterium avium* subsp. *paratuberculosis*. *Vet Res.* (2021) 52:69. doi: 10.1186/S13567-021-00940-Y

10. Juste RA, Perez V. Control of paratuberculosis in sheep and goats. *Vet Clin North Am Food Anim Pract.* (2011) 27:127–38. doi: 10.1016/j.cvfa.2010.10.020

11. Corpa JM, Pérez V, García Marín JF, Sánchez MA. Control of paratuberculosis (Johne's disease) in goats by vaccination of adult animals. *Vet Record.* (2000) 146:195–6. doi: 10.1136/vr.146.7.195

12. Corpa JM, Garrido J, García Marín JF, Pérez V. Classification of lesions observed in natural cases of paratuberculosis in goats. *J Comp Pathol.* (2000) 122:255–65. doi: 10.1053/jcpa.1999.0368

13. Perez V, Marin JFG, Badiola JJ. Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep. *J Comp Pathol.* (1996) 114:107–22. doi: 10.1016/S0021-9975(96)80001-6

14. Fernández M, Royo M, Fuertes M, Arteche-Villasol N, Ferreras MC, Benavides J, Pérez V. Effects of paratuberculosis vaccination at different ages in a dairy goat herd: a 2-year follow-up. *Animals.* (2022) 12:3135. doi: 10.3390/ani12223135

15. Mercier P, Brémaud I, Gautier MP. Vaccination of kids under one month of age with a killed vaccine and reduction in the frequency of faecal shedding of *Mycobacterium avium* subspecies *paratuberculosis*. *Small Ruminant Research.* (2014) 121:425–33. doi: 10.1016/j.smallrumres.2014.09.007

16. Eppleston J, Windsor P. Lesions attributed to vaccination of sheep with Gudair^{TM} for the control of ovine paratuberculosis: post farm economic impacts at slaughter. *Aust Vet J.* (2007) 85:129–33. doi: 10.1111/j.1751-0813.2007.00135.x

17. Bastida F, Juste RA. Paratuberculosis control: a review with a focus on vaccination. *J Immune Based Ther Vaccines.* (2011) 9:8. doi: 10.1186/1476-8518-9-8

18. Bezos J, de Juan L, Romero B, Álvarez J, Mazzucchelli F, Mateos A, et al. Experimental infection with *Mycobacterium caprae* in goats and evaluation of immunological status in tuberculosis and paratuberculosis co-infected animals. *Vet Immunol Immunopathol.* (2010) 133:269–75. doi: 10.1016/j.vetimm.2009.07.018

19. Gilardoni LR, Paolicchi FA, Mundo SL. Diagnosis of bovine paratuberculosis 201 Bovine paratuberculosis: a review of the advantages and disadvantages of different diagnostic tests. *Rev Argent Microbiol.* (2012) 44:201–15.

20. Castellanos E, Juan L de, Domínguez L, Aranaz A. Progress in molecular typing of *Mycobacterium avium* subspecies *paratuberculosis*. *Res Vet Sci.* (2012) 92:169–79. doi: 10.1016/j.rvsc.2011.05.017

21. Pillai SR, Jayarao BM. Application of IS900 PCR for detection of Mycobacterium avium subsp. *paratuberculosis* directly from raw milk. *J Dairy Sci.* (2002) 85:1052–7. doi: 10.3168/jds.S0022-0302(02)74165-9

22. Whittington R, Donat K, Weber MF, Kelton D, Nielsen SS, Eisenberg S, et al. Control of paratuberculosis: Who, why and how. A review of 48 countries. *BMC Vet Res.* (2019) 15:198. doi: 10.1186/s12917-019-1943-4

23. Krüger C, Köhler H, Liebler-Tenorio EM. Sequential development of lesions 3, 6, 9, and 12 months after experimental infection of goat kids with *Mycobacterium avium* subsp *paratuberculosis*. *Vet Pathol.* (2015) 52:276–90. doi: 10.1177/0300985814533804

24. Uzal FA, Plattner BL, Hostetter JM. Chapter 1-Alimentary system. In: Maxie MG, editor. *Jubb, Kennedy & Palmer's Pathology of Domestic Animals.* 6th ed. Philadelphia, PA: W.B. Saunders (2016). p. 1–257.e2.

25. Khodakaram Tafti A, Rashidi K. The pathology of goat paratuberculosis: gross and histopathological lesions in the intestines and mesenteric lymph nodes. *J Vet Med Series B.* (2000) 47:487–95. doi: 10.1046/j.1439-0450.2000.00375.x

26. Whittington RJ, Begg DJ, de Silva K, Purdie AC, Dhand NK, Plain KM. Case definition terminology for paratuberculosis (Johne's disease). *BMC Vet Res.* (2017) 13:6. doi: 10.1186/s12917-017-1254-6

27. Benavides J, González L, Dagleish M, Pérez V. Diagnostic pathology in microbial diseases of sheep or goats. *Vet Microbiol.* (2015) 181:15–26. doi: 10.1016/j.vetmic.2015.07.012

28. Kheirandish R, Tafti AK, Hosseini A. Classification of lesions and comparison of immunohistochemical and acid-fast staining in diagnosis of naturally occurring paratuberculosis in goats. *Small Ruminant Res.* (2009) 87:81–5. doi: 10.1016/j.smallrumres.2009.09.030

29. Kurade NP, Tripathi BN, Rajukumar K, Parihar NS. Sequential development of histologic lesions and their relationship with bacterial isolation, fecal shedding, and immune responses during progressive stages of experimental infection of lambs with *Mycobacterium avium* subsp. *paratuberculosis*. *Vet Pathol.* (2004) 41:378–87. doi: 10.1354/vp.41-4-378

30. Jenvey CJ, Hostetter JM, Shircliff AL, Bannantine JP, Stabel JR. Quantification of macrophages and *Mycobacterium avium* subsp. *paratuberculosis* in bovine intestinal tissue during different stages of johne's disease. *Vet Pathol.* (2019) 56:671–80. doi: 10.1177/0300985819844823

31. González J, Geijo M V, García-Pariente C, Verna A, Corpa JM, Reyes LE, et al. Histopathological classification of lesions associated with natural paratuberculosis infection in cattle. *J Comp Pathol.* (2005) 133:184–96. doi: 10.1016/J.JCPA.2005.04.007

32. Bezos J, Roy Á, Infantes-Lorenzo JA, González I, Venteo Á, Romero B, et al. The use of serological tests in combination with the intradermal tuberculin test maximizes the detection of tuberculosis infected goats. *Vet Immunol Immunopathol.* (2018) 199:43–52. doi: 10.1016/j.vetimm.2018.03.006

33. Roy A, Tomé I, Romero B, Lorente-Leal V, Infantes-Lorenzo JA, Domínguez M, et al. Evaluation of the immunogenicity and efficacy of BCG and MTBVAC vaccines using a natural transmission model of tuberculosis. *Vet Res.* (2019) 50:82. doi: 10.1186/s13567-019-0702-7

34. King JM, Roth-Johnson L, Dodd DC, Newsom ME. *The Necropsy Book: A Guide for Veterinary Students, Residents, Clinicians, Pathologists, and Biological Researchers*. Ithaca, NY: The Internet-Fist University Press (2014)

35. MAPA. *National Bovine Tuberculosis Eradication Program 2021*. (2021). Available online at: http://pa.gob.es/es/ganaderia/temas/sanidad-animalhigieneganadera/programatb2021versionabril_tcm30%E2%80%93561045.pdf (accessed July 8, 2024).

36. Wangoo A, Johnson L, Gough J, Ackbar R, Inglut S, Hicks D, et al. Advanced granulomatous lesions in *Mycobacterium bovis*-infected cattle are associated with increased expression of type I procollagen, γδ (WC1+) T cells and CD 68+ cells. *J Comp Pathol.* (2005) 133:223–34. doi: 10.1016/j.jcpa.2005.05.001

37. Prophet EB. *Laboratory Methods in Histotechnology*. Washington, DC: American Registry of Pathology. (1994).

38. Espeschit IF, Schwarz DGG, Faria ACS, Souza MCC, Paolicchi FA, Juste RA, et al. Paratuberculosis in Latin America: a systematic review. *Trop Anim Health Prod.* (2017) 49:1557–76. doi: 10.1007/s11250-017-1385-6

39. Kravitz A, Pelzer K, Sriranganathan N. The paratuberculosis paradigm examined: a review of host genetic resistance and innate immune fitness in *Mycobacterium avium* subsp. *paratuberculosis* infection. *Front Vet Sci.* (2021) 8:721706. doi: 10.3389/fvets.2021.721706

40. Sonawane GG, Tripathi BN. Comparative evaluation of diagnostic tests for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in the tissues of sheep affected with distinct pathology of paratuberculosis. *Int J Mycobacteriol.* (2016) 5:S88–S89. doi: 10.1016/j.ijmyco.2016.09.005

41. Seva J, Sanes JM, Ramis G, Mas A, Quereda JJ, Villarreal-Ramos B, et al. Evaluation of the single cervical skin test and interferon gamma responses to detect *Mycobacterium bovis* infected cattle in a herd co-infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Vet Microbiol.* (2014) 171:139–46. doi: 10.1016/j.vetmic.2014.03.035

42. Mortier RAR, Barkema HW, De Buck J. Susceptibility to and diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in dairy calves: a review. *Prev Vet Med.* (2015) 121:189–98. doi: 10.1016/j.prevetmed.2015.08.011

43. Koets A, Ravesloot L, Ruuls R, Dinkla A, Eisenberg S, Lievaart-Peterson K. Effects of age and environment on adaptive immune responses to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) vaccination in dairy goats in relation to paratuberculosis control strategies. *Vet Sci.* (2019) 6:62. doi: 10.3390/VETSCI6030062

44. Stefanova EP, Quesada-Canales Ó, Paz-Sánchez Y, Caballero MJ, Quintana-Montesdeoca del P, Espinosa de los Monteros A, et al. Morphological assessment of concomitant lesions detected in goat herds naturally infected with paratuberculosis (Johne's disease). *Animals.* (2023) 13:1693. doi: 10.3390/ani13101693

45. Smith MC, Sherman DM. *Goat Medicine*. Hoboken: John Wiley & Sons. (2009).

46. Matthews JG. Chapter 9 – Chronic weight loss. In: *Diseases of the Goat*. Hoboken: John Wiley & Sonsp (2016). p. 115–130.

47. Gutiérrez Cancela MM, García Marín JF. Comparison of Ziehl-Neelsen staining and immunohistochemistry for the detection of *Mycobacterium bovis* in bovine and caprine tuberculous lesions. *J Comp Pathol.* (1993) 109:361–70. doi: 10.1016/S0021-9975(08)80299-X

48. Valheim M, Storset AK, Aleksersen M, Brun-Hansen H, Press CML. Lesions in subclinical paratuberculosis of goats are associated with persistent gut-associated lymphoid tissue. *J Comp Pathol.* (2002) 127:194–202. doi: 10.1053/jcpa.2002.0581

49. Stewart DJ, Vaughan JA, Stiles PL, Noske PJ, Tizard MLV, Prowse SJ, et al. long-term bacteriological and immunological study in Holstein-Friesian cattle experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis* and necropsy culture results for Holstein-Friesian cattle, Merino sheep and Angora goats. *Vet Microbiol.* (2007) 122:83–96. doi: 10.1016/j.vetmic.2006.12.030

50. Thoresen OF, Falk K, Evensen Ø. Comparison of immunohistochemistry, acid-fast staining, and cultivation for detection of *Mycobacterium paratuberculosis* in goats. *J Vet Diagnost Investig.* (1994) 6:195–99.

51. Martinson SA, Hanna PE, Ikede BO, Lewis JP, Miller LM, Keefe GP, et al. Comparison of bacterial culture, histopathology, and immunohistochemistry for the diagnosis of Johne's disease in culled dairy cows. *J Vet Diagn Invest.* (2008) 20:51–7. doi: 10.1177/104063870802000109

52. Kheirandish R, Khodakaram-Tafti A, Hosseini A. The comparative evaluation of immunohistochemical and acid-fast staining with histopathological changes in naturally occurring paratuberculosis in sheep. *Comp Clin Path.* (2008) 17:111–6. doi: 10.1007/s00580-007-0696-9

53. Hailat NQ, Hananeh W, Metekia AS, Stabel JR, Al-Majali A, Lafi S. Pathology of subclinical *paratuberculosis* (Johne's disease) in Awassi sheep with reference to its occurrence in Jordan. *Veterinarni Medicina.* (2010) 55: 590–602. doi: 10.17221/2947-VETMED

54. Plante Y, Remenda BW, Chelack BJ, Haines DM. Detection of *Mycobacterium paratuberculosis* in formalin-fixed paraffin-embedded tissues by the polymerase chain reaction. *Can J Vet Res.* (1996) 60:115.

55. Derakhshandeh A, Namazi F, Khatamsaz E, Eraghi V, Hemati Z. Goat *paratuberculosis* in Shiraz: Histopathological and molecular approaches. *Vet Res Forum.* (2018) 9:253–7. doi: 10.30466/vrf.2018.32080

56. Conde C, Price-Carter M, Cochard T, Branger M, Stevenson K, Whittington R, Bannantine JP, Biet F. Whole-genome analysis of *Mycobacterium avium* subsp. *paratuberculosis* IS900 insertions reveals strain type-specific modalities. *Front Microbiol.* (2021) 12:660002. doi: 10.3389/fmicb.2021.660002

CAPÍTULO III

Prevalencia serológica de la paratuberculosis caprina y respuesta inmune a la vacunación frente a Mycobacterium avium subespecie paratuberculosis en las Islas Canarias, España

7. CAPÍTULO III

7.1. RESUMEN

Prevalencia serológica de la paratuberculosis caprina y respuesta inmune a la vacunación frente a Mycobacterium avium subespecie paratuberculosis en las Islas Canarias, España

Caprine paratuberculosis seroprevalence and immune response to anti-Mycobacterium avium subspecies paratuberculosis vaccination on the Canary Islands, Spain

La paratuberculosis (PTB), causada por *Mycobacterium avium* subespecie *paratuberculosis* (MAP), es una enfermedad crónica que afecta a rumiantes domésticos y salvajes. Este estudio se realizó en 12 granjas caprinas de las Islas Canarias, que tienen la cuarta mayor población caprina de España y están oficialmente indemnes de tuberculosis bovina (TB). Se realizaron dos sesiones de muestreo con un total de 2.774 muestras de suero, analizadas mediante un ensayo inmunoabsorbente ligado a enzimas (ELISA, enzyme linked immunosorbent assay). En la primera sesión, la prevalencia global fue del 18,4%, variando entre el 2,5% y el 61,1%. Se establecieron 3 grupos de prevalencia inicial: A, (0-10%]; B, de (10-20%] y C, >20%. Se demostraron diferencias estadisticamente significativas entre las granjas del grupo C, sin tener en cuenta la edad de los animales (p=0,001). Sin embargo, una vez analizada la edad, se confirmaron diferencias significativas en los tres grupos de prevalencia tanto entre el número de positivos en cada rango de edad, como entre las proporciones. En cuanto al análisis de las caracteristicas de cada granja, solamente se demostraron diferencias significativas en el grupo C en cuanto a la seropositividad y la temperatura media anual (p=0,001), la presencia de granjas en los alrededores (p=0,001) y la presencia de vallado (p=0,001). En la segunda sesión, se evaluó el efecto de la vacunación frente a MAP con una vacuna comercial inactivada (Gudair®), incluyendo animales vacunados (V) y no vacunados (nV). Se registraron diferentes tendencias de desarrollo de anticuerpos en granjas en distintos grupos de prevalencia inicial. En las granjas del grupo A, más cabras adultas desarrollaron anticuerpos tras la vacunación. En las granjas de grupo B y C, la respuesta a la vacunación fue heterogénea. Sin embargo, únicamente en el grupo C se demostraron diferencias significativas y proporcionales entre la seroconversión en los distintos grupos de edad.

Este trabajo caracteriza la situación de la PTB en las Islas Canarias y aporta nuevos conocimientos sobre el efecto de la prevalencia de la granja en la respuesta inmune a la vacunación contra PTB, aunque se necesita futuros estudios incluyendo más granjas. La vacunación reduce, pero no elimina, la excreción fecal de MAP, los casos clínicos y las lesiones en los órganos afectados. Doce (12) meses post vacunación, se observaron variaciones en los niveles de anticuerpos en función de la prevalencia inicial y la edad de los animales. Los resultados sugieren que la seroprevalencia puede estar relacionada con la edad y las características ambientales de la granja, pero también destacan la importancia de futuras investigaciones para evaluar otros factores que influyen en la respuesta inmunitaria y la epidemiología de la PTB caprina.

Este estudio proporciona una visión inicial sobre la prevalencia de la PTB y sus variaciones entre granjas en las Islas Canarias, destacando la importancia del monitoreo serológico continuo y las medidas de bioseguridad para controlar la propagación de la enfermedad. Además de la necesidad de combinar ELISA con otras técnicas, para una mejor evaluación del estado de la enfermedad en las granjas afectadas.

veterinary sciences

IMPACT FACTOR 2.0

Indexed in: PubMed

CITESCORE 2.9

Article

Caprine Paratuberculosis Seroprevalence and Immune Response to Anti-*Mycobacterium avium* Subspecies *paratuberculosis* Vaccination on the Canary Islands, Spain

Elena Plamenova Stefanova, Yania Paz-Sánchez, Óscar Quesada-Canales, María del Pino Quintana-Montesdeoca, Antonio Espinosa de los Monteros, Ana Sofía Ramírez, Antonio Fernández and Marisa Andrada

Special Issue
Immunological Assessment of Veterinary Infectious Diseases

Edited by
Prof. Dr. María José García-Iglesias, Prof. Dr. César-B. Gutiérrez Martín and Dr. Sonia Martinez

MDPI

https://doi.org/10.3390/vetsci11090388

veterinary sciences

MDPI

Article

Caprine Paratuberculosis Seroprevalence and Immune Response to Anti-*Mycobacterium avium* Subspecies *paratuberculosis* Vaccination on the Canary Islands, Spain

Elena Plamenova Stefanova [1,2,*], Yania Paz-Sánchez [1,2,*], Óscar Quesada-Canales [1,2], María del Pino Quintana-Montesdeoca [3], Antonio Espinosa de los Monteros [1,2], Ana Sofía Ramírez [4], Antonio Fernández [1,2] and Marisa Andrada [1,2]

1 Veterinary Histology and Pathology, Institute of Animal Health and Food Safety (IUSA), Veterinary School, University of Las Palmas de Gran Canaria, 35413 Arucas, Gran Canaria, Spain; oscar.quesada@ulpgc.es (Ó.Q.-C.); antonio.espinosa@ulpgc.es (A.E.d.l.M.); antonio.fernandez@ulpgc.es (A.F.); marisaana.andrada@ulpgc.es (M.A.)

2 Departament of Morphology, Veterinary School, University of Las Palmas de Gran Canaria, 35413 Arucas, Gran Canaria, Spain

3 Departament of Mathematics, University of Las Palmas de Gran Canaria, 35017 Las Palmas de Gran Canaria, Gran Canaria, Spain; mariadelpino.quintana@ulpgc.es

4 Epidemiology and Preventive Medicine Unit, Institute of Animal Health and Food Safety (IUSA), Veterinary School, University of Las Palmas de Gran Canaria, 35413 Arucas, Gran Canaria, Spain; anasofia.ramirez@ulpgc.es

* Correspondence: elena.plamenova101@alu.ulpgc.es (E.P.S.); yania.paz102@gmail.com (Y.P.-S.)

Simple Summary: Paratuberculosis (PTB) is a chronic disease that affects domestic and wild ruminants worldwide. This study was conducted in 12 diary caprine farms on the Canary Islands. The region counts with the fourth largest goat population in Spain and has "officially free" bovine tuberculosis status. Two sampling sessions were conducted, and 2774 serum samples were tested by an enzyme-linked immunosorbent assay. In the first session, a prevalence of 18.4% was obtained, varying from 2.5% up to 61.1%. In the second session, the effect of PTB vaccination was evaluated and both non-vaccinated (nV) and vaccinated (V) were included. Variable tendencies in antibody development were registered in farms with different initial seroprevalences. In farms in which up to 10% of the animals were positive, more adult goats had antibodies against PTB after vaccination. In farms with more than 10% of ELISA-positive animals, a heterogeneous response to vaccination was reported. We observed that in farms with higher initial prevalence, fewer goats that were V developed antibodies. Our work characterizes the caprine PTB situation on the Canary Islands and gives new insights on the effect of farm prevalence on the immune response to PTB vaccination, although further studies on a greater scale are needed.

Abstract: Paratuberculosis (PTB), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic disease with economic impact on ruminant farming worldwide. The Canary Islands count with the fourth largest goat population in Spain and are "officially free" of bovine tuberculosis. Twelve farms were included with 2774 serum samples tested by an enzyme-linked immunosorbent assay (ELISA) for detection of anti-MAP antibodies in two sessions. In the first session, an overall apparent prevalence of 18.4% (2.5% up to 61.1%) was obtained. Farms with prevalences (0–10%], (10–20%] and >20% were identified, with differences in seroconversion in the same prevalence group between farms and age ranges. Non-vaccinated (nV) and vaccinated (V) animals were included in the second sampling session. Higher levels of antibodies were detected in V animals older than 12 months, with considerable variations between age ranges and farms. Our results describe the current PTB status of the Canary Islands' goat farming. Furthermore, new insights on the effect of the farm prevalence on seroconversion in V animals are provided, although further studies are needed to evaluate the multiple factors affecting the immune response to anti-MAP vaccination.

check for updates

Citation: Stefanova, E.P.; Paz-Sánchez, Y.; Quesada-Canales, Ó.; Quintana-Montesdeoca, M.d.P.; Espinosa de los Monteros, A.; Ramírez, A.S.; Fernández, A.; Andrada, M. Caprine Paratuberculosis Seroprevalence and Immune Response to Anti-*Mycobacterium avium* Subspecies *paratuberculosis* Vaccination on the Canary Islands, Spain. *Vet. Sci.* **2024**, *11*, 388. https://doi.org/10.3390/vetsci11090388/

Academic Editors: María José García-Iglesias, Sonia Martinez and César-B. Gutiérrez Martín

Received: 30 June 2024
Revised: 12 August 2024
Accepted: 22 August 2024
Published: 23 August 2024

Keywords: ELISA; Johne's disease; goat; antibody; serological test; age; mycobacteria

1. Introduction

Paratuberculosis (PTB), also known as Johne´s disease, is a chronic wasting disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) that affects both domestic and wild ruminants worldwide [1–6]. Animals are usually infected at a young age by ingestion of fecal material present in a contaminated environment, water, or food, as well as by drinking milk or colostrum from infected adult animals [3,4,6]. Intrauterine infection has also been described [1,3,4,6].

Although animals get infected at a young age, the subclinical phase is long, and clinical signs such as watery diarrhea, weight loss, and reduction in milk production appear only in the terminal phase [7,8]. Furthermore, the clinical signs in small ruminants are not as straightforward as in cattle [3]. In goats, data about the effect of age on clinical signs development are limited. Nevertheless, infected ruminants can eliminate MAP in their feces from initial stages of infection, which makes early diagnosis very important for correct control and prevention of the disease in affected areas [3–5].

PTB is a World Organization for Animal Health (WOAH)-listed disease and must be reported to this organization as indicated in the Terrestrial Animal Health Code [9]. Furthermore, according to the renewed animal health European Union (EU) legislation (Regulation (EU) 2018/1882), PTB is listed as a 'category E disease' for which there is a need for surveillance within the Union, as referred to in Article 9(1)(e) of Regulation (EU) 2016/429. The definitive confirmation method includes post-mortem identification of PTB-compatible lesions and histopathological examination [1–3,10]. Nevertheless, early ante-mortem diagnosis is hampered by the relatively low sensitivity of diagnostic tests on an individual level and the lack of pathognomonic signs, which leads to well-established infection in the herd before the first case is diagnosed [3,4,6,11].

Control programs usually include immunodiagnostic tests such as the enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion (AGID) assay, intradermal skin testing, lymphocyte transformation, and IFNγ assays [3,5,12]. Polymerase chain reaction (PCR) targeting the insertion sequence 900 (IS 900) in fecal samples is a frequently used technique for herd screening as it has proven to be one of the most sensitive methods for MAP DNA detection. Microbiological culture, on the other hand, is a gold standard for PTB confirmation, although it is timely, and its detection capacity is limited in the early stages of PTB [12,13].

Furthermore, vaccination is used as a control tool as it has proven to reduce but not eliminate MAP fecal shedding, clinical cases, and lesions in target organs of affected animals [11,12,14–16]. Nevertheless, anti-MAP vaccination is banned in some countries, such as Denmark, and requires a special authorization in France, Germany, and Spain [12]. Only some countries apply PTB vaccination on goats, including Australia, Spain, and the Netherlands [12]. In Spain, vaccination in some regions, such as the Canary Islands, is subject to special authorization from the local authorities due to its interference with tuberculosis (TB) diagnosis. Although data about the seroconversion of vaccinated goats is scarce, some authors suggest that it might be related to various factors, including the age of vaccination and the environmental MAP dose to which animals are exposed on the farm [17,18].

However, the use of anti-MAP vaccines is controversial as it has been proven to interfere in the interpretation of intradermal skin tests, which are used in eradication programs of mycobacterial infections such as bovine tuberculosis (TB) [11,12,16].

In recent years, goat production has increased worldwide, mainly in developing countries, due to the low input it requires [5,6,19]. The small ruminant industry has significantly contributed to the alleviation of poverty in Africa and Asia [5]. Currently, Europe bares the third-largest goat production [20]. Spain has the second largest population on the continent, with a total of 2.293 million heads (2023) [21], distributed mainly between 5 autonomous communities. Andalucía counts with 37% of the population, Castilla-La Mancha with 15%, Extremadura with 10%, the Canary Islands with 10.3%, and Murcia with 9.7% [20]. Data about PTB prevalence, however, are limited, except for Andalucia, where a recent study established a seroprevalence of 20% [4]. Nevertheless, the disease is considered widespread in continental Spain, originating considerable economic losses [4,22].

The present study was conducted on the Canary Islands, which counts with the fourth largest Spanish goat population with a total of 200,054 heads and 1256 farms [23] of mainly certified autochthonous endangered breeds (Orden APM/26/2018). Most of the goat population is centered on the islands of Fuerteventura and Gran Canaria, which count with 73,572 and 47,388 heads, respectively [23]. Since the region was granted an "officially free" status for TB in 2017, vaccination against PTB is subject to a special protocol established by the local authorities (Decreto 51/2018 del 23 de abril) [24]. The aim of our work is to evaluate the current status of PTB on the Canary Islands throughout ELISA-based seropositivity measurement and the effect of vaccination on the humoral immune response in naturally infected herds.

2. Materials and Methods

2.1. Study Design and Sampling

2.1.1. Animals and Farms

A total of 12 dairy caprine farms (5 from Fuerteventura and 7 from Gran Canaria) were sampled between 2018 and 2022. All animals were Majorera goats, a local certified autochthonous endangered breed (Orden APM/26/2018). All samples were submitted to the Institute of Animal Health and Food Safety (IUSA), Veterinary School, University of Las Palmas de Gran Canaria, as part of the official request process for an anti-MAP vaccination permit. In all farms, the presence of PTB was suspected by the identification of clinical signs, including severe emaciation, protrusion of lumbar vertebrae, easily palpable transverse processes, muscle mass loss, and a reduction in visceral fat deposits.

The sampling was conducted in 2 sessions, and a total of 2774 serum samples were analyzed, sampling with a minimum expected prevalence of 10% and a 95% CI. In the first sampling session, approximately 15% of the census of each farm was tested. Subsequently, 7 farms (3 from Fuerteventura and 4 from Gran Canaria) were granted an authorization for vaccination against PTB. A second sampling session was conducted 12 months after the first session in 9/12 farms, and 1274 serum samples were analyzed. Both vaccinated (V) and non-vaccinated (nV) animals were included in the second sampling session as the local legislation specifies that a control group of nV animals should be left on farms that are granted a vaccination permit (Artículo 4 del Decreto 51/2018 del 23 de abril) [24]. The number of serum samples per farm and sampling session is summarized in Table 1.

2.1.2. "TB-Free" Status Confirmation

This study was conducted on the Canary Islands, in which PTB vaccination is regulated by the Decreto 51/2018 del 23 de abril [24], which marks the requirements to obtain a vaccination permit. Since the islands are "officially free" of bovine tuberculosis, farmers who wish to implement anti-MAP vaccination need to certify that the animals are free of tuberculosis and that PTB is present in the farm [25,26]. As part of this process, all farms that requested vaccination were subjected to an on-field comparative intradermal tuberculin (CIT) test for detection of the *Mycobacterium tuberculosis* complex. All animals with positive or inconclusive results were send to slaughter. The absence of tuberculosis in those was confirmed by histopathology and bacterial cultures performed by the laboratory of VISAVET, Health Surveillance Centre, Madrid, Spain. All farms included certified that they were officially TB-free.

Table 1. Number of caprine serum samples per farm and island and PTB herd confirmation techniques.

Farm	1st Sampling	2nd Sampling			PTB Herd Confirmation
		V	nV	Total	
Fuerteventura	1098	165	769	934	
F1	36	36	34	70	+
F2	740	117	623	740	++
F3	80	12	68	80	++
F4	196	-	-	-	+
F5	46	0	44	44	+
Gran Canaria	402	200	140	340	
F6	46	-	-	-	+
F7	43	30	12	42	+
F8	38	79	36	115	+
F9	15	20	10	30	+
F10	91	71	25	96	+
F11	19	-	-	-	+
F12	150	0	57	57	++
Total	1500	365	909	1274	

F, farm; V, vaccinated; nV, non-vaccinated. + Includes post-mortem necropsy/slaughterhouse sampling with gross and/or histopathological granulomatous lesions in mesenteric lymph nodes and/or ileocecal valve and Ziehl–Neelsen and/or immunohistochemistry-positive samples. ++ Includes post-mortem PTB confirmation and PCR-positive tissue samples for IS900 identification.

2.1.3. PTB Confirmation on Herd Level

In all farms included in this study, PTB was confirmed on herd level by post-mortem examination by necropsy performance and/or sampling at slaughter in all farms [25,26]. Afterwards, histopathological identification of granulomatous lesions affecting the mesenteric lymph nodes and/or the ileocecal valve was performed, as well as Ziehl–Neelsen for identification of acid-fast bacteria and/or immunohistochemistry for identification of MAP antigens [25]. In 3 farms, an additional real-time polymerase chain reaction (PCR) for MAP DNA detection targeting the insertion sequence 900 (IS900) was performed on tissue samples [25]. Details about the PTB confirmation techniques used in each farm are summarized in Table 1.

2.2. Serum Sampling

The whole blood samples were obtained by puncture of the jugular vein using sterile tubes without anticoagulant (Vacutainer®, Becton-Dickinson, Franklin Lakes, NJ, USA). Subsequently, those were transferred to the laboratory under refrigeration within the first 24 h after the sampling. Afterwards, the blood was centrifuged at 400 g for 10 min, and serum was obtained. Samples were stored at −20 °C until the analysis was performed.

2.3. Anti-MAP Vaccine

The anti-MAP vaccine applied was 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. One milliliter of vaccine was subcutaneously administered in the post-scapular area of the back 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. In the herds that were granted vaccination permission from the local authorities, a control group of nV animals was left as required by the local legislation (Decreto 51/2018 del 23 de abril) [24]. These goats were managed under the same conditions as the V ones.

2.4. Serological Assay

Serum samples were analyzed using a commercial in vitro diagnostic ELISA test kit for detection of antibodies to *Mycobacterium avium* subspecies *paratuberculosis* (*Mycobacterium paratuberculosis* Test Kit PARACHEK® 2, Prionics AG, Schlieren, Switzerland) following the manufacturer's protocol. According to the data provided in the data sheet, the sensitivity (Se) in goats ranges from 65 to 88%, and the specificity (Sp) is of 99% or greater.

2.5. Farm Characterization

An official geographic tool designed by the local authorities on the Canary Islands (Sistema de Información Territorial de Canarias© GRAFCAN 1989-2024) was used to collect data on the production characteristics of the farms. Information about the following general production and biosecurity variables was extracted for each farm: altitude, distance to a main road, presence of other farms in a perimeter of 1 km^2, livestock perimeter fencing, and mean annual temperature.

2.6. Prevalence Groups

Farms were categorized in the following groups based on the within-herd apparent prevalence: group A, farms with (0–10%] seropositivity; group B, (10–20%] seropositivity; and group C, with ≥20% seropositivity.

2.7. Statistical Analysis

An observational cross-section study was conducted. The apparent seroprevalence was calculated separately on farm level. The true seroprevalence was calculated using the Se and Sp of the kit used, using the lowest Se value indicated by the manufacturer (65%).

Statistical analysis of data was performed by IBM SPSS Statistics 27 (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA: IBM Corp). The age was summarized using the mean, standard deviation (SD), median, and interquartile range (IQR). Shapiro–Wilk and Kolmogorov–Smirnov tests were used to analyze the age and mean annual temperature normality. A non-parametric Mann–Whitney U and Kruskal–Wallis tests were used to compare the means of two independent samples (age/mean annual temperature and ELISA results). Categorical variables were summarized using percentages and relative or absolute frequencies. The ages of the studied goats were categorized in the following groups: (0–12) months, [12–24) months, [24–36) months, [36–48) months, [48–60) months, and ≥60 months. A chi-square test was used to contrast the association between two categorical variables. Additionally, the Bonferroni correction for multiple testing was applied to control experiment-wise and family-wise error rates.

The results were considered statistically significant if the p-value < 0.05.

3. Results

3.1. First Sampling Session

3.1.1. Seroprevalence

The overall apparent individual seroprevalence was 18.4% (257/1500). The within-herd seroprevalence varied from 2.5% up to 61.1%, and this difference was statistically significant between the farms ($p = 0.001$). In all farms, positive animals were detected. The Se and Sp were used to calculate the true prevalence. The individual true prevalence was 27.19%, and the within-herd antibody detection ranged from 2.34% to 93.92%.

Subsequently, three prevalence groups were established for further analyses: group A with farms with (0–10%]; group B with (10–20%], and group C with >20%. In group A, 5 farms with a total of 379 animals were included. In group B, 3 farms with 256 animals were sampled. In group C, 4 farms with 865 goats were evaluated. The farms within-herd seroprevalence of each farm are summarized in Table 2.

Table 2. Within-farm apparent and true seroprevalence and farm characterization.

Farm	Apparent Seroprevalence (%)	True Seroprevalence (%)	1st Sampling Session (Month)	Fencing (Y/N)	Altitude (m)	Mean Annual Temperature (°C)	Distance to a Main Road (km)	Ruminant Farms in the Surrounding Area (Y/N)
Group A (0–10%]								
F3	2.5	2.3	May	Y	<600	21.2	<1	Y
F4	9.7	13.6	May	Y	<600	21.4	>1	N
F6	6.5	8.6	October	Y	<600	20.4	<1	Y
F8	2.6	2.6	October	Y	<600	20.6	<1	Y
F11	5.3	6.7	October	Y	<600	20.7	0	Y
Group B (10–20%]								
F9	20	29.7	October	Y	>600	15	0	N
F10	12.1	17.3	October	N	<600	20.1	0	Y
F12	16.7	24.5	March	Y	<600	20.2		Y
Group C > 20%								
F1	61.1	93.9	October	N *	<600	19.4 *	<1	Y *
F2	22.4	33.5	June	Y *	<600	21.1 *	<1	Y *
F5	21.7	32.4	January	Y *	<600	20.2 *	>1	N *

F, farm; Y, yes; N, no; m, meter; km, kilometer; °C, degree Celsius. * Statistically significant associations with $p < 0.05$.

3.1.2. Age Analysis

The animals sampled in the first session were between 1 and 210 months old, with a mean of 20.44 months, a median of 12 months, a SD of 17.03 months, and an IQR of 12 months. The goats were included in the following age groups: 128 had (0–12) months, 962 had [12–24) months, 172 had [24–36) months, 102 had [36–48) months, 81 had [48–60) months, and 44 had ≥60 months of age. No information was available about the age of 11 goats. Thus, 64.6% of the studied animals had between [12 and 24) months (19.9% positive and 80.1% negative).

In group A, no statistical differences were present between the within-farm ELISA results regardless the age of the animals (p = 0.200) (Table 3). Nevertheless, statistical differences were demonstrated between the ELISA results in the different age ranges (p = 0.002) with proportional differences in the group of (0–12) with 0% of positive animals and [24–36) with 14.92% of positive goats (Figure 1a). Within each age range, no differences were detected between farms. Furthermore, the proportions in each age range were separately assessed and no differences were demonstrated (Figure 1b).

In group B, the differences between the farms, regardless of the age, were not significant (p = 0.549) (Table 3). Nevertheless, statistical differences were demonstrated between the results in the different age ranges (p = 0.001) with proportional differences in the animals from [12–24) and [24–36) months of age with 8% and 35.9% of positive goats, respectively (Figure 1c). Once the results in the different age ranges were compared between the farms, differences were demonstrated in the predominant age group of [12–24) months (p = 0.001) in which the ELISA-positive animals in the three farms were 100%, 2%, 17.4%, respectively. However, no proportional differences were detected (Figure 1d).

In group C, statistical differences between the four farms in relation to the number of positive and negative animals were demonstrated regardless of the age of the animals (p = 0.001) (Table 3). Once the age of the animals was assessed without taking into account the farm of origin, the differences were also significant (p = 0.014), with proportional differences being present in the groups of (0–12), [12–24), and [26–48) months with 0%, 23.3%, and 44% of seropositivity, respectively (Figure 1e). Furthermore, association between the within-farm results was also confirmed in the age group of [12–24) months (p = 0.001), in which farms the seroprevalences were of 0%, 16.7%, 22.4%, and 64.7%, with a significant proportional difference being present (Figure 1f).

Table 3. Statistical associations between within-farm ELISA results in caprine farms from the same prevalence group.

Farm	1st Sampling Session		2nd Sampling Session			
			V		nV	
	ELISA + (%)	p-Value [1]	ELISA + (%)	p-Value [1]	ELISA + (%)	p-Value [1]
Group A (0–10%]						
F3 F4 F6 F8 F11	2/80; 2.5% 19/196; 9.7% 3/46; 6.5% 1/38; 2.6% 1/19; 5.3%	0.200	7/12; 58.3% - - 70/79; 88.6% -	**0.007**	33/68; 48.5% - - 0/36; 0% -	**0.001**
Group B (10–20%]						
F9 F10 F12	3/15; 20% 11/91; 12.1% 25/150; 16.7%	0.549	17/20; 85% 40/71; 56.3% -	**0.019**	2/10; 20% 19/25; 76% 5/57; 8.8%	**0.001**
Group C > 20%						
F1 F2 F5 F7	22/36; 61.1% 166/740; 22.4% 10/46; 21.7% 13/43; 30.2%	**0.001**	25/36; 69.4% 48/117; 41% - 26/30; 86.7%	**0.001**	0/34; 0% 149/623; 23.9% 7/44; 15.9% 7/12; 58.3%	**0.001**

[1] Chi-square test. The results were considered statistically significant if $p < 0.05$ (**bold values**).

3.1.3. Farm Characterization

The farm characterization (Table 2) carried out demonstrated that the majority of the herds included in this study were completely fenced, with only 2/12 being not fenced. Statistical differences between ELISA results and the presence of fencing were not significant in neither group A, in which all farms were fenced, nor in group B (p = 0.298). In group C, 61.1% of the animals from non-fenced farms were positive in contrast with 22.8% from farms with complete fencing and the differences were significant (p = 0.001). The average altitude was of <600 m, with 2/12 farms being situated on higher altitudes. No statistical differences between the ELISA results and the altitude of the farms were detected in group A, in which all farms were situated on <600 m, group B (p = 0.597), or group C (p = 0.360). The mean annual temperature ranged from 15 °C to 21.40 °C with 2/12 farms presenting less than 19 °C. Significant associations between the ELISA results and the mean annual temperature were not detected, neither in group A (p = 0.062) nor group B (p = 0.597). In group C, F1 and F7 presented the highest seroprevalences and the lowest mean annual temperatures, and the differences were significant (p = 0.001). Regarding the distance to a main road, only 2/12 farms was situated on >1 km from the closest main road. The differences between the ELISA results and the distance to the main road were not significant in group A (p = 0.075), group B (p = 0.448), or group C (p = 0.612). Lastly, only 3/12 farms did not have any other ruminant farm in the surrounding area. The ELISA result had no significant association with the surrounding farms in group A (p = 0.064) or group B (p = 0.597). In group C, 21.7% of positivity was registered in herds with no other farms in the surrounding area and 24.2% in herds with other ruminant farms present in the surroundings, and the difference was significant (p = 0.001).

Figure 1. Overall and within-farm seroprevalence of the first sampling session per age range and prevalence group. Each bar pattern (P1 and P2) denotes a subset of within-farm ELISA result categories in the same age range whose column proportions do not differ significantly from each other at the 0.05 level (Bonferroni correction). (**a**) Overall seroprevalence in farms from group A (0–10%]

per age group; (**b**) within-farm seroprevalence in farms from group A (0–10%]; (**c**) overall seroprevalence in farms from group B (10–20%] per age group; (**d**) within-farm seroprevalence in farms from group B (10–20%]. Statistical differences were demonstrated between farms in the age group [12–24) months, * (p = 0.001). (**e**) Overall seroprevalence in farms from group C (>20%) per age group; (**f**) within-farm seroprevalence in farms from group C (>20%). Statistical differences were demonstrated between farms in the age groups [12–24) months * (p = 0.001).

3.2. Second Sampling Session

A total of 1274 samples from 9/12 farms were tested 12 months after the first sampling session. Overall, a total of 35.1% (455/1274) of the animals tested positive. In the V group, 63.8% (233/365) goats tested positive, and in the nV, 24.4% (222/909).

Age Analysis

The age of the animals in the second sampling session ranged from 6 to 213 months, with a mean of 21.67 months, a median of 12 months, a SD of 21.13 months, and an IQR of 14 months. The number of goats in each age group was as follows: 409 had (6–12) months, 513 had [12–24) months, 143 had [24–36) months, 71 had [36–48) months, 39 had [48–60) months, and 99 had ≥60 months of age. Thus, 72.4% of the studied animals were between 6 and 24 months of age, of which 29.2% tested positive (72.9% nV and 27.1% V) and 70.8% were negative (89.3% nV and 10.7% V).

Furthermore, the differences between the positive and negative samples were analyzed separately in the V and nV animals in each prevalence group.

In group A, a total of 195 animals from 2/5 of the originally sampled farms were included. A difference between the number of positive and negative animals in the farms was demonstrated in both V (p = 0.007) and nV (p = 0.001) goats, regardless of the age range (Table 3). In the case of the nV animals, this difference was confirmed in the age groups of (6–12) and [12–24) months in which one of the farms had no positive animals and in the other one 40% (p = 0.001) and 55.3% (p = 0.035) of the goats presented anti-MAP antibodies, respectively. Once the proportions in each age range were separately assessed, no differences were demonstrated. In the case of the V animals, no statistical differences were demonstrated between the ELISA results in the different age ranges (p = 0.928), although a higher percentage of positive animals was detected in older animals (Figure 2a). It is worth highlighting that no animals 6–12 months old were present in the farms from this group, and thus the immune response in young goats could not be evaluated. On the other hand, within each age range, differences between the farms were detected in the predominant age group of [12–24) months in which the 92.6% of the V goats from the first farm tested positive in contrast with 58.3% in the second farm (p = 0.010), although no proportional differences were detected (Figure 2b). No statistical analysis was performed in the other age groups, as only one farm had animals older than 24 months, but a tendency was observed of a higher percentage of seroconversion in older animals.

In the farms from group B, the same three farms included in the first sampling were checked, with a total of 183 sera obtained. Differences between the number of positive and negative goats were detected between both V (p = 0.019) and nV (p = 0.001) animals, regardless of the age range (Table 3). Regarding the nV animals, differences were present in the age range of [12–24) months in which the three farms presented a seroprevalence of 16.7%, 20%, and 76%, respectively (p = 0.002). Nevertheless, no proportional differences were demonstrated between the farms in this age range. In the case of the V goats, no statistical differences were demonstrated between the ELISA results in the different age ranges (p= 0.071), although seropositivity tended to decrease in older animals (Figure 2c). It is worth mentioning that no animals 6–12 months old were present in the farms from this group, and thus the immune response in young goats could not be evaluated. On the other hand, differences between the within-farms' seropositivity were only demonstrated in the age group of [24–36) months in which one of the farms had only positive V animals in contrast with the other one in which only 64.7% of the goats presented anti-MAP antibodies

($p = 0.021$). The proportions, on the other hand, did not significantly differ (Figure 2d). The third farm did not implement vaccination as a control tool and thus had no V goats. In general, the seroconversion among the V animals from different farms was heterogeneous, being the lowest percentage of animals with anti-MAP antibodies from F10, in which the highest percentage of nV-positive animals was detected.

Figure 2. Overall and within-farm seroprevalence of the second sampling session per age range and prevalence group in anti-MAP-vaccinated (V) goats. Each bar pattern (P1 and P2) denotes a subset of within-farm ELISA result categories in the same age range whose column proportions do not differ

significantly from each other at the 0.05 level (Bonferroni correction). (**a**) Overall seroconversion in V animals from group A (0–10%] farms per age range with a tendency of higher antibody levels in adult goats; (**b**) within-farm seroprevalence in V animals from farms in prevalence group A (0–10%] with statistical differences between farms in age group [12–24) months * $p = 0.010$ and higher percentage of seroconversion in older animals; (**c**) overall seroconversion in V animals from group B (10–20%] farms per age range with a tendency of higher antibody levels in younger goats; (**d**) within-farm seroprevalence in V animals from farms in prevalence group B (10–20%] with statistical differences between farms in the age group of [24–36) months * $p = 0.021$ and a general heterogeneous anti-MAP vaccination response in the different farms; (**e**) overall seroconversion in V animals from group C (>20%) farms per age range with proportional differences and lowest seroconversion in animals (6–12) months old and higher in [24–36) old goats; (**f**) within-farm seroprevalence in V animals from farms in prevalence group C (>20%). Statistical differences were demonstrated between animals ≥ 60 months, * $p = 0.041$, and a heterogeneous immune response to anti-MAP vaccination between farms.

Finally, in group C, all four originally tested farms were checked with a total of 896 samples. In one of them, vaccination was not implemented. A difference between the number of positive and negative samples in the different farms was demonstrated in the group of the V ($p = 0.001$) and the nV ($p = 0.001$) animals regardless of the age of the animals (Table 3). Regarding the nV animals, differences were demonstrated in the age range (6–12) and [12–24) months with $p = 0.001$ and $p = 0.013$, respectively. In the first age group, only F2 of the farms had positive goats (24.3%), and this proportional difference was statistically significant in comparison with F1 and F5, which had no positive animals. In the second age group, one of the farms presented 16.7% seropositivity in contrast with 22% and 58.3% in the other two, and the proportional difference was statistically significant at the 0.05 level. The last farm had no nV animals in this age group. Regarding the V goats, statistical differences were demonstrated between the ELISA results in the different age ranges ($p = 0.001$). The lowest seroconversion levels were registered in the animals (6–12) months old with 20% and the highest in the age range of [24–36) months with 80.2% of seropositivity (Figure 2e). Significant proportional differences were also confirmed at 0.05 level. Regarding the within-farm seropositivity, in the three farms in which vaccination was implemented, differences were confirmed only between the oldest animals ($\geq$60 months), in which in one of the farms 75% of the V goats presented anti-MAP antibodies in contrast with 41.6% and 90% in the other two ($p = 0.041$). This proportional difference was statistically significant at the 0.05 level (Figure 2f). The farm with the lowest level of seroconversion in this age group was F1, which is the farm with the highest seroprevalence in the first sampling (61.1%). In general, the seroconversion, although not statistically significant, was heterogeneous among the different farms.

4. Discussion

Paratuberculosis is a well-known problem in ruminant farms worldwide and is also a WOAH-listed disease and thus must be notified as marked by the Terrestrial Animal Health Code [9]. Nevertheless, a review published in 2019 suggested that in 74% of the countries in which PTB was notifiable, it was underreported [12]. In Spain, various studies have been published stating that PTB is a widespread problem [4,6,22]. Although, in the second half of 2022, only 224 cases were reported in ovine and caprine species in Spain and none of them in the Canary Islands [27].

Furthermore, PTB's prevalence is considered to be underestimated regardless of the geographic location [4,12]. The main reasons for this situation included in a recent report are as follows: low sensitivity of the diagnostic test, lack of surveillance, and lack of knowledge or awareness on the clinical signs of the disease [12]. In Spain, various reports have been published in order to estimate the prevalence of the disease in different regions of the country [4,6,28]. To the authors' knowledge, this is the first study reporting the current caprine PTB status of the Canary Island archipelago, which bears the fourth largest goat population in Spain [20]. The overall apparent individual seroprevalence of 18.4% found in our work is slightly lower than two previous descriptions conducted in the region of Andalucia, which carries the largest goat population in the country. These reports detected goat-positive serum samples in 20% and 22.5% [4,6]. It is worth mentioning that the sensitivity of the ELISA is relatively low, mainly in the early stages of the disease, and thus, as mentioned in other studies, the true PTB prevalence might be underestimated [3,4,6,12,29].

On the other hand, biosecurity measures are considered one of the most effective preventive strategies against PTB spread in a herd and between herds [3–5,7,8,30]. Although data about exact risk factors in goat farms is limited, a previous study conducted in Spain shows that the risk of seropositivity to MAP was 2.2 times higher in farms without full perimeter fencing [4]. In our work, the highest herd prevalence was found in a non-fenced farm with other ruminant farms nearby (F1, 61.1%). Furthermore, the statistical differences between the number of positive and negative animals in the farms from group C in relation to the fencing, the mean annual temperature, and the presence of other ruminant farms in the surrounding area suggest the important role of the farm characteristics and biosecurity measures on the animals' anti-MAP immune response and PTB status [3,17].

Furthermore, the age of the affected animals was analyzed in detail, as it is a well-known fact that the incubation phase of PTB is long [3,5,12,13]. However, data about how long a goat can shed MAP and exhibit no clinical signs are scarce. In sheep, a review study stated that usually clinical PTB is detected in animals older than 2 years, with many being older than 4 years [31]. In cattle, cases of asymptomatic infected cows of up to 14 years have been described [31]. In our study, 64.6% of the studied animals had between 12 and 24 months of age, which can be explained by the standard productive age range in caprine farming. Furthermore, the results of the present work show a tendency of higher immune response being detected in older animals, regardless of the prevalence group of the farms. In a recent study from a naturally infected farm with relatively low prevalence, Fernandez et al. detected higher initial antibody levels in the adult animals older than 1.5 years from the non-vaccinated control group in contrast with the animals younger than 6 months [15]. Another study from Mercier et al. in heavily infected farms detected an increase in the seropositivity of the control group of goat kids 15.5 months after the beginning of the study and even higher levels once 23 months had passed [32]. Furthermore, in all prevalence groups, animals older than ≥60 months were present, and a considerable part tested positive. Those animals could be potential shedders of the disease into the environment and, given the relatively low Se of the ELISA test, their number might be underestimated [4–6,33,34]. Thus, our results highlight the importance of future studies analyzing the role of keeping old animals in PTB-affected farms on the prevalence and dissemination of the disease in the herd. On the other hand, the statistical differences demonstrated in the main age groups from the included farms with similar prevalences confirm that, as suggested by other authors, anti-MAP antibody development is multifactorial and not strictly age-related. In naturally infected herds, doses and infection routes are different, and the disease stages of the included animals can vary and should be considered [3,5,31,34].

Lastly, this work evaluates the effect of age on seroconversion once anti-MAP vaccination is implemented. Numerous studies have demonstrated the beneficial effects of PTB vaccination on the reduction in clinical cases and histopathological lesions in affected herds [5,11,14,15,35]. Nevertheless, vaccination implementation does not prevent infection; although MAP shedding in vaccinated animals is reduced, those can still eliminate MAP into the environment and stay infectious [11,12,14,36]. Based on those facts and following the manufacturer's instructions, in heavily infected herds, both young and adult animals were vaccinated. In our work, 63.8% of the V animals developed anti-MAP antibodies with variable seroconversion tendencies in the different prevalence groups. In group A, in which the farms had relatively low initial prevalence, a clear tendency of higher seropositivity in older animals was observed. Similar results were observed in group C, which was the only one in which animals between 6 and 12 months could be evaluated, and those were the ones with lower antibody levels. These results are in line with a study published by

Corpa et al. that showed that the antibody response was higher in animals vaccinated at 5 months of age in contrast with those 15 days old [18]. The authors' hypothesis states that the effect on antibody development might be explained by the fact that the immune system is mature in older animals in contrast with goat kids. In group B, however, the tendency was for a decrease in the antibody levels in older animals. Nevertheless, that tendency was not identic in the two farms evaluated. Furthermore, although it was not statistically demonstrated, farms with initial higher seroprevalences had fewer V ELISA-positive goats. A recent study suggested that the presence of anti-MAP antibodies in vaccinated animals might be related to the prevalence of environmental MAP in the farm they are raised in and is not strictly related to the age of vaccination [17]. Furthermore, in our work, the farm with higher seroprevalence (F1, 61.1%), presented the lowest percentage of animals $\geq$ 60 months of age that developed anti-MAP antibodies. Previous studies of mycobacterial infections, including the BCG vaccine in humans, have highlighted the possibility of elevated presence of mycobacteria in the environment to be the cause of blocking or masking the immune response in adult animals with matured immune system that have been continuously exposed to MAP [15].

Finally, it is worth highlighting that non-vaccinated animals were included in the second sampling, and although the levels of anti-MAP antibodies were similar, a seroprevalence of 24.4% was registered in contrast with the initial 18.4% in the first sampling. These results demonstrate the importance of serological surveillance in order to detect, assess, and control PTB in affected herds even after vaccination, as it does not prevent infection nor eliminate MAP shedding [5,12,31,37]. Nevertheless, ELISA tests, although widely used and cost-effective, still have limitations such as low sensitivity and the impossibility to differentiate between vaccinated and infected animals [12,13,16–18]. Thus, serological screening should be combined with other widely used MAP detection techniques, such as fecal PCR targeting IS 900, to better assess PTB herd status [12,13].

5. Conclusions

The present study describes the current epidemiological situation of PTB on the Canary Islands, which holds the fourth largest goat population in Spain. We demonstrate that PTB is endemic on the islands, with an average apparent prevalence of 18.4% varying from 2.5% up to 61.1% between the farms included. Furthermore, we conclude that the age of the animals might be related to anti-MAP antibody development, with higher seroprevalences being detected in animals older than 12 months. On the other hand, we demonstrate that age might not be the only factor affecting seroconversion, as considerable differences are observed between farms from different prevalence groups and with variable biosecurity and environmental characteristics, which suggest the possible role of the environment on seroconversion. Nevertheless, animals $\geq$ 60 months old are present in most of the farms, and a considerable percentage of them are ELISA-positive. Thus, we highlight the importance of test-and-cull practices implementation for reduction in possible MAP-shedders and correct assessment of the disease on farm level. This work also shows that the use of ELISA serological tests to diagnose PTB is a useful tool and should be implemented as part of the control protocol in the farms from the Canary archipelago. Moreover, we analyze the effect of vaccination, demonstrating the differences between the seroconversion in young and adult animals. We illustrate that in some cases, young animals do not seroconvert as expected after vaccination. Also, we observe that the amount of MAP present in the herd might be related to the level of antibodies developed after vaccination. Nevertheless, further studies are needed to explore the effect of the environmental component as well as other risk factors on the epidemiology and pathogenesis of caprine PTB.

Author Contributions: Conceptualization, Y.P.-S., Ó.Q.-C., A.F. and M.A.; methodology, E.P.S., Y.P.-S., Ó.Q.-C., M.d.P.Q.-M., A.S.R. and M.A.; validation, E.P.S., Y.P.-S. and M.A.; formal analysis, Y.P.-S., E.P.S. and M.A.; investigation, E.P.S., Y.P.-S., Ó.Q.-C., A.E.d.l.M. and M.A.; resources, A.E.d.l.M., A.F. and M.A.; data curation, E.P.S., A.S.R., M.d.P.Q.-M. and M.A.; writing—original draft preparation, E.P.S., Y.P.-S. and M.A.; writing—review and editing, E.P.S., Y.P.-S., Ó.Q.-C., M.d.P.Q.-M., A.F.,

A.E.d.l.M. and M.A.; visualization, E.P.S., Y.P.-S., Ó.Q.-C. and M.A.; supervision, A.E.d.l.M., A.S.R. and M.A.; project administration, E.P.S., Y.P.-S., A.E.d.l.M., A.F. and M.A.; funding acquisition, A.E.d.l.M., A.F. and M.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the 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. Elena Plamenova Stefanova was the recipient of a predoctoral contract of the Ministry of Universities, Spain (FPU20/03693) and Charter100 2021 scholarship for PhD students' mobility.

Institutional Review Board Statement: Ethical review and approval were waived for this study by the Ethical Committee for Animal Experimentation of the University of Las Palmas of Gran Canaria (OEBA-CEEA-ULPGC- 13/2023), which notified that the procedure falls within the exceptions; thus, 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 Ethical Committee for Animal Experimentation.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

Acknowledgments: The authors thank the veterinarians from the Livestock Health Protection Groups responsible for the management of the farms for providing the animals for this study, post-mortem technician Javier Ramos, the laboratory technicians of Institute of Animal Health and Food Safety (IUSA) Ana Afonso Almeda and Alba María Santana Zerpa for their technical assistance, and the veterinary technicians from the local authorities represented by Gestión del Medio Rural de Canarias, Gobierno de Canarias for their collaboration in the process of sera collection.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Uzal, F.A.; Plattner, B.L.; Hostetter, J.M. Chapter 1—Alimentary System. In *Jubb, Kennedy & Palmer's Pathology of Domestic Animals: Volume 2*, 6th ed.; Maxie, M.G., Ed.; W.B. Saunders: Philadelphia, PA, USA, 2016; pp. 1–257.e2, ISBN 978-0-7020-5318-4.
2. Benavides, J.; González, L.; Dagleish, M.; Pérez, V. Diagnostic Pathology in Microbial Diseases of Sheep or Goats. *Vet. Microbiol.* **2015**, *181*, 15–26. [CrossRef] [PubMed]
3. Windsor, P.A. Paratuberculosis in Sheep and Goats. *Vet. Microbiol.* **2015**, *181*, 161–169. [CrossRef] [PubMed]
4. Jiménez-Martín, D.; García-Bocanegra, I.; Risalde, M.A.; Fernández-Molera, V.; Jiménez-Ruiz, S.; Isla, J.; Cano-Terriza, D. Epidemiology of Paratuberculosis in Sheep and Goats in Southern Spain. *Prev. Vet Med.* **2022**, *202*, 105637. [CrossRef] [PubMed]
5. Idris, S.M.; Eltom, K.H.; Okuni, J.B.; Ojok, L.; Elmagzoub, W.A.; El Wahed, A.A.; Eltayeb, E.; Gameel, A.A. Paratuberculosis: The Hidden Killer of Small Ruminants. *Animals* **2022**, *12*, 12. [CrossRef]
6. Barrero-Domínguez, B.; Luque, I.; Huerta, B.; Gomez-Laguna, J.; Galán-Relaño, Á.; Gómez-Gascón, L.; Sánchez, M.; Astorga, R.J. Paratuberculosis in Dairy Goat Flocks from Southern Spain: Risk Factors Associated with Seroprevalence. *Vet Rec.* **2019**, *185*, 600. [CrossRef]
7. Matthews, J. Herd Health and Biosecurity. In *Diseases of the Goat*; John Wiley & Sons: Hoboken, NJ, USA, 2016; pp. 339–345, ISBN 9781119073543.
8. Smith, M.C.; Sherman, D.M. *Goat Medicine*; John Wiley & Sons: Hoboken, NJ, USA, 2009; ISBN 0781796431.
9. Terrestrial Animal Health Code. WOAH. Available online: https://www.woah.org/en/what-we-do/standards/codes-and-manuals/terrestrial-code-online-access/ (accessed on 21 June 2024).
10. González, J.; Geijo, M.V.; García-Pariente, C.; Verna, A.; Corpa, J.M.; Reyes, L.E.; Ferreras, M.C.; Juste, R.A.; García Marín, J.F.; Pérez, V. Histopathological Classification of Lesions Associated with Natural Paratuberculosis Infection in Cattle. *J. Comp. Pathol.* **2005**, *133*, 184–196. [CrossRef]
11. Bastida, F.; Juste, R.A. Paratuberculosis Control: A Review with a Focus on Vaccination. *J. Immune Based Ther. Vaccines* **2011**, *9*, 8. [CrossRef]
12. Whittington, R.; Donat, K.; Weber, M.F.; Kelton, D.; Nielsen, S.S.; Eisenberg, S.; Arrigoni, N.; Juste, R.; Sáez, J.L.; Dhand, N.; et al. Control of Paratuberculosis: Who, Why and How. A Review of 48 Countries. *BMC Vet Res.* **2019**, *15*, 198. [CrossRef]
13. Gilardoni, L.R.; Paolicchi, F.A.; Mundo, S.L. Diagnosis of Bovine Paratuberculosis: A Review of the Advantages and Disadvantages of Different Diagnostic Tests. *Rev. Argent Microbiol.* **2012**, *44*, 201–215.

14. Corpa, J.M.; Pérez, V.; García Marín, J.F.; Sánchez, M.A. Control of Paratuberculosis (Johne's Disease) in Goats by Vaccination of Adult Animals. *Vet. Rec.* **2000**, *146*, 195–196. [CrossRef]
15. Fernández, M.; Royo, M.; Fuertes, M.; Arteche-Villasol, N.; Ferreras, M.C.; Benavides, J.; Pérez, V. Effects of Paratuberculosis Vaccination at Different Ages in a Dairy Goat Herd: A 2-Year Follow-Up. *Animals* **2022**, *12*, 3135. [CrossRef] [PubMed]
16. Espinosa, J.; Fernández, M.; Royo, M.; Grau, A.; Ángel Collazos, J.; Benavides, J.; del Carmen Ferreras, M.; Mínguez, O.; Pérez, V. Influence of Vaccination against Paratuberculosis on the Diagnosis of Caprine Tuberculosis during Official Eradication Programmes in Castilla y León (Spain). *Transbound. Emerg. Dis.* **2020**, *68*, 692–703. [CrossRef] [PubMed]
17. Koets, A.; Ravesloot, L.; Ruuls, R.; Dinkla, A.; Eisenberg, S.; Lievaart-Peterson, K. Effects of Age and Environment on Adaptive Immune Responses to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) Vaccination in Dairy Goats in Relation to Paratuberculosis Control Strategies. *Vet. Sci.* **2019**, *6*, 62. [CrossRef]
18. Corpa, J.M.; Pérez, V.; García Marín, J.F. Differences in the Immune Responses in Lambs and Kids Vaccinated Against Paratuberculosis, According to the Age of Vaccination. *Vet. Microbiol.* **2000**, *77*, 475–485. [CrossRef] [PubMed]
19. Schwarz, D.G.G.; Lima, M.C.; Barros, M.; Valente, F.L.; Scatamburlo, T.M.; Rosado, N.; Oliveira, C.T.S.A.M.; Oliveira, L.L.; Moreira, M.A.S. Short Communication: Passive Shedding of *Mycobacterium avium* Ssp. *paratuberculosis* in Commercial Dairy Goats in Brazil. *J. Dairy Sci.* **2017**, *100*, 8426–8429. [CrossRef]
20. El Sector de Ovino y Caprino en Cifras, © *Ministerio de Agricultura, Pesca y Alimentación, NIPO: 003-22-136-X*. Available online: https://www.mapa.gob.es/es/agricultura/estadisticas/indicadoreseconomicosdelsectorovinoycaprinodeleche2023_nuevasactualizaciones_tcm30-109075.pdf (accessed on 21 June 2024).
21. Resultados de las Encuestas de Ganado Ovino y Caprino, © *Ministerio de Agricultura, Pesca y Alimentación, NIPO: 003-22-136-X*. Available online: https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias/ganaderia/encuestas-ganaderas/ (accessed on 21 June 2024).
22. de Juan Ferré, L.; Domínguez Rodríguez, L.; Suárez Fernández, G.; Mateos García, A. *Paratuberculosis Caprina: Aportaciones a Su Diagnóstico, Epidemilogía Molecular y Control*; Universidad Complutense de Madrid: Madrid, Spain, 2005; ISBN 8466928529.
23. Censo Ganadero, ISTAC 2023. Available online: https://www3.gobiernodecanarias.org/istac/statistical-visualizer/visualizer/data.html?resourceType=indicator&resourceId=GANADO_CAPRINO&measure=ABSOLUTE&geo=ES70#visualization/table (accessed on 21 June 2024).
24. Decreto 51/2018 del 23 de Abril. Available online: https://www.gobiernodecanarias.org/boc/2018/084/001.html (accessed on 21 June 2024).
25. Stefanova, E.P.; Sierra, E.; Fernández, A.; Quesada-Canales, O.; Paz-Sánchez, Y.; Colom-Rivero, A.; Espinosa de los Monteros, A.; Herráez, P.; Domínguez, L.; Bezos, J.; et al. Detection of Caprine Paratuberculosis (Johne's Disease) in Pre- and Post-Vaccinated Herds: Morphological Diagnosis, Lesion Grading, and Bacterial Identification. *Front. Vet Sci.* **2024**, *11*, 1395928. [CrossRef]
26. Stefanova, E.P.; Quesada-Canales, Ó.; Paz-Sánchez, Y.; Caballero, M.J.; Quintana-Montesdeoca, M.D.P.; Espinosa de los Monteros, A.; Rivero, M.A.; Castro, A.; Pérez, V.; Andrada, M. Morphological Assessment of Concomitant Lesions Detected in Goat Herds Naturally Infected with Paratuberculosis (Johne's Disease). *Animals* **2023**, *13*, 1693. [CrossRef]
27. Informe Semestral Para La Notificación de Las Enfermedades, Infecciones e Infestaciones de La Lista de La OIE, Segundo trimestre-2022. *Organización mundial de sanidad animal (OIE) © 2023 OIE via Ministerio de Agricultura, Pesca y Alimentación*. Available online: https://www.mapa.gob.es/es/ganaderia/temas/sanidad-animal-higiene-ganadera/smr_160123_terrestrial_14042023_espana_tcm30-619669.pdf (accessed on 21 June 2024).
28. Falconi, C.; Oleaga, Á.; López-Olvera, J.R.; Casais, R.; Prieto, M.; Gortázar, C. Prevalence of Antibodies against Selected Agents Shared between Cantabrian Chamois (*Rupicapra Pyrenaica Parva*) and Domestic Goats. *Eur. J. Wildl. Res.* **2010**, *56*, 319–325. [CrossRef]
29. Whittington, R.J.; Begg, D.J.; de Silva, K.; Purdie, A.C.; Dhand, N.K.; Plain, K.M. Case Definition Terminology for Paratuberculosis (Johne's Disease). *BMC Vet. Res.* **2017**, *13*, 328. [CrossRef]
30. Gaffuri, A.; Barsi, F.; Magni, E.; Bergagna, S.; Dellamaria, D.; Ricchi, M.; De Paolis, L.; Galletti, G.; Arrigoni, N.; Lorenzi, V.; et al. Paratuberculosis, Animal Welfare and Biosecurity: A Survey in 33 Northern Italy Dairy Goat Farms. *Animals* **2023**, *13*, 2346. [CrossRef]
31. Whittington, R.J.; Sergeant, E. Progress towards Understanding the Spread, Detection and Control of *Mycobacterium avium* subsp *paratuberculosis* in Animal Populations. *Aust. Vet. J.* **2001**, *79*, 267–278. [CrossRef]
32. Mercier, P.; Brémaud, I.; Gautier, M.P. Vaccination of Kids under One Month of Age with a Killed Vaccine and Reduction in the Frequency of Faecal Shedding of *Mycobacterium avium* Subspecies *paratuberculosis*. *Small Rumin. Res.* **2014**, *121*, 425–433. [CrossRef]
33. Chaubey, K.K.; Gangwar, N.; Pawaiya, R.S.; Jatav, G.P.; Sohal, J.S.; Singh, S.V.; Singh, M.; Gupta, S.; Kumaresan, G.; Kumar, N.; et al. Evaluation of Newly Developed 'Six Recombinant Secretary Proteins Based "Cocktail ELISA" and "Whole Cell Lysate" Based "Indigenous ELISA" and Tissue Microscopy' with 'Gold Standard' Histo-Pathology for the Diagnosis of Johne's Disease in Slaughtered Goats. *Comp. Immunol. Microbiol. Infect. Dis.* **2019**, *66*, 101338. [CrossRef] [PubMed]
34. Nielsen, S.S.; Toft, N. Ante Mortem Diagnosis of Paratuberculosis: A Review of Accuracies of ELISA, Interferon-γ Assay and Faecal Culture Techniques. *Vet. Microbiol.* **2008**, *129*, 217–235. [CrossRef]

35. Aguilló-Ros, I.; Andrada, M.; Pérez-Sancho, M.; Roy, Á.; Bezos, J.; Bonnet, T.; Moreno, I.; Paz-Sánchez, Y.; Domínguez, M.; Gómez-Villamandos, J.C.; et al. Effect of Heat-Inactivated *Mycobacterium avium* subspecies *paratuberculosis* (MAP) Vaccine on the Lesions and Immunopathology Developed in Target Tissues of Naturally MAP-Infected Goats. *Vet. Microbiol.* **2022**, *273*, 109543. [CrossRef] [PubMed]
36. Juste, R.A.; Perez, V. Control of Paratuberculosis in Sheep and Goats. *Vet. Clin. N. Am. Food Anim. Pract.* **2011**, *27*, 127–138. [CrossRef]
37. Muskens, J.; van Zijderveld, F.; Eger, A.; Bakker, D. Evaluation of the Long-term Immune Response in Cattle After Vaccination Against Paratuberculosis in Two Dutch Dairy Herds. *Vet. Microbiol.* **2002**, *86*, 269–278. [CrossRef]

DISCUSIÓN

8. DISCUSIÓN

Los tres trabajos resaltan varios aspectos críticos relacionados con el diagnóstico de la PTB en infección natural, tanto en animales con enfermedad clínica, como subclínica.

Desafíos Diagnósticos y Técnicas Utilizadas

Uno de los temas centrales es la dificultad para diagnosticar la PTB de manera precoz debido a su largo periodo subclínico. En los tres estudios, se destaca que los métodos de diagnóstico ampliamente utilizados, como el análisis serológico mediante ELISA o la PCR para la detección de ADN de MAP en tejidos, presentan limitaciones. Sin embargo, estos métodos son esenciales para controlar la diseminación de la enfermedad en los rebaños caprinos, dado que la PTB tiende a propagarse antes de la manifestación de los síntomas clínicos evidentes que en el caprino se reducen a la pérdida de peso, ya que la diarrea es poco frecuente.

La confirmación de la enfermedad en los rebaños se apoya también en el examen postmortem y el análisis histopatológico de lesiones granulomatosas en LN MS y la VIC, así como en pruebas adicionales como la tinción de ZN e IHQ. Los resultados reflejan la variedad de lesiones que se pueden detectar en animales afectados y destacan la importancia de la integración del examen anatomopatológico, sobre todo la confirmación histológica, como prueba rutinaria para la detección temprana de la enfermedad ya que las lesiones de PTB se detectaron tanto en casos clínicos como subclínicos.

Impacto de la Vacunación

Otro aspecto clave discutido es el uso de la vacunación como herramienta de control. La vacunación reduce la eliminación fecal de MAP y disminuye la aparición de casos clínicos, aunque no elimina por completo la infección. En los estudios se muestra cómo la vacunación en animales jóvenes y adultos puede llevar a la seroconversión (desarrollo de anticuerpos detectables), pero los resultados son heterogéneos dependiendo de factores como la edad del animal en el momento de la vacunación y la prevalencia inicial de MAP en la granja.

Los datos indican que, en granjas con baja prevalencia inicial de la enfermedad, los animales adultos tienden a desarrollar una respuesta inmunitaria más robusta, mientras que en granjas con alta prevalencia, los niveles de anticuerpos después de la vacunación son menos consistentes. Esto podría estar relacionado con la adaptación del sistema inmunológico debido a la exposición constante al patógeno en ambientes altamente contaminados.

Por otro lado, se confirma el efecto de la vacunación sobre la disminución de lesiones granulomatosas tanto a nivel de LN MS como en VIC en casos clínicos y subclínicos. Además, los resultados demuestran que, aunque no elimina la infección, la vacunación reduce cuantitativamente la carga de MAP en órganos diana. Por último, la vacunación también podría tener efecto beneficioso generalizado en el rebaño, especialmente en las patologías concomitantes de origen inflamatorio, principalemnte a nivel respiratorio. Sin embargo, hacen falta más futuros estudios para confirmar esta hipótesis.

Factores Ambientales y Bioseguridad

Los tres artículos subrayan también la importancia de las medidas de bioseguridad en la prevención de la PTB. Se observó que las granjas con medidas más estrictas, como cercas y mayor distancia a otras granjas, tendían a tener una menor prevalencia de la enfermedad.

Además, los factores ambientales, como la temperatura anual media, aun en un clima estable como el de Canarias, parecen influenciar la propagación de la PTB. Esto sugiere que las condiciones ambientales podrían influir en la respuesta inmunitaria frente a la infección de MAP.

Conclusión

En conjunto, los artículos proporcionan una visión integral del estado actual de la PTB caprina en las Islas Canarias y los desafíos asociados a su control. Se destaca la necesidad de combinar diferentes métodos diagnósticos y valorar las estrategias de control como la implementación de vacunación junto con estrictas medidas de bioseguridad de acuerdo a la situación epidemiológica en el rebaño. Sin embargo, aún se necesitan estudios adicionales para comprender mejor el impacto de factores ambientales y de manejo en la respuesta inmunitaria y la propagación de la infección.

CONCLUSIONES

9. CONCLUSIONES/CONCLUSIONS

1. El examen histopatológico es importante para el diagnóstico correcto de la paratuberculosis caprina tanto en casos clínicos como subclínicos, ya que mediante el examen macroscópico no se pueden detectar ni la linfadenitis granulomatosa con formación de microgranulomas, ni la enteritis granulomatosa leve y multifocal no calcificada.

   Histopathological examination is important for correct diagnosis of caprine PTB in both clinical and subclinical cases as by gross examination neither granulomatous lymphadenitis with microgranuloma formation, nor mild and multifocal noncalcified granulomatous enteritis can be detected.

2. Las patologías concomitantes más comunes encontradas en rebaños caprinos con infección natural de PTB en las Islas Canarias son de origen inflamatorio y afectan principalmente a los sistemas hemolinfático, respiratorio y gastrointestinal.
   The most common concomitant pathologies found in goat herds with natural PTB infection on the Canary Islands are of inflammatory origin and mainly affect the hemolyphnatic, respiratory and gastrointestinal systems.

3. La vacunación contra MAP podría tener un efecto beneficioso en la reducción de patologías concomitantes como los procesos inflamatorios respiratorios.

   Vaccination against MAP might have a beneficial effect on the reduction of concomitant pathologies such as respiratory inflammation processes.

4. Es recomendable utilizar una combinación de técnicas de laboratorio que incluyan Zielh-Neelsen, inmunohistoquímica y PCR dirigida contra IS900 para la correcta identificación etiológica de MAP en cabras en infección natural, ya que en casos con baja carga bacteriana la concordancia entre las tres técnicas varía de baja a moderada.

   It is advisable to use a combination of laboratory techniques including Zielh-Neelsen, immunohistochemistry and PCR targeting IS900 for correct etiological identification of MAP in naturally infected goats, as in cases with low bacterial load the agreement between the three techniques varies from low to moderate.

5. La vacunación contra MAP puede mitigar la gravedad de la enteritis granulomatosa a lesiones leves multifocales y reducir la carga bacteriana en los órganos diana, pero no previene completamente la infección. Los linfonodos mesentéricos, tanto en casos clínicos como subclínicos vacunados y no vacunados, suelen presentar granulomas encapsulados de grado IV con necrosis central y mineralización.

   Vaccination against MAP can mitigate the severity of the granulomatous enteritis to mild multifocal lesions and reduce the bacterial load in target organs but cannot fully prevent infections. Mesenteric lymph nodes in both vaccinated and non-vaccinated clinical and subclinical cases mostly present stage IV encapsulated granulomas with central necrosis and mineralization.

6. La PTB es endémica en las Islas Canarias, con una prevalencia aparente promedio del 18,4% y variaciones considerables entre las explotaciones afectadas. En granjas con >20% de seroprevalencia inicial, características como la temperatura media anual, el vallado y la proximidad a otras explotaciones podrían influir en el desarrollo de anticuerpos.

   PTB is endemic on the Canary Islands, with an average apparent prevalence of 18.4% with considerable variations between the affected farms. In farms with >20% initial seroprevalence, characteristics such as mean annual temperature, fencing and proximity to other farms could also play a role in antibody development.

7. El desarrollo de anticuerpos anti-MAP podría estar relacionado con la edad de los animales, aunque pueden existir diferencias entre los grupos de edad en rebaños con prevalencias iniciales similares.

   The anti-MAP antibody development might be related to the age of the animals, although differences can exist between the age groups in herds with similar initial prevalences.

8. Existen diferencias en la seroconversión después de la vacunación entre animales jóvenes y adultos. Aunque algunas cabras jóvenes parecen no seroconvertir según lo esperado, en explotaciones con >10% de seroprevalencia la respuesta inmunitaria es heterogénea entre los grupos de edad.

There are differences between the seroconversion after vaccination between young and adult animals. Although some young goats seem to not seroconvert as expected, in farms with >10% seroprevalence the immune response is heterogeneous among the age groups.

9. Para diseñar un protocolo de detección temprana y evaluación de casos naturales de PTB caprina en animales vacunados y no vacunados, se debe considerar una combinación de diferentes herramientas que incluyan el examen postmortem, pruebas de laboratorio para la identificación del agente y el control serológico.

   To design a protocol for early detection and assessment of natural caprine PTB cases in vaccinated and non-vaccinated animals, a combination of different tools including postmortem examination, laboratory tests for agent identification and serological control should be considered.

AGRADECIMIENTOS
Acknowledgments
Благодарности

10. AGRADECIMIENTOS/ acknowledgments/благодарности

“Si he visto más lejos, es poniéndome sobre los hombros de gigantes” dijo Isaac Newton. Partiendo de esta gran verdad, procedo a agradecer a mis gigantes por orden cronológico.

На първо място, благодаря на семейството си. Без вас никога нямаше да стигна до края на това приключение, нито пък щях да го започна. Благодаря ви за всички безсънни нощи, за всички сълзи, които проронихте с мен и за мен, за безграничната подкрепа, опора и най-вече за примера, който ми дадохте. Благодаря, че ме научихте на най-важното: каквото и където и да става, винаги заедно продължаваме напред. И тъй като така и не станах писателка, посвещавам на вас и специално на баба Елена/д-р Попова, този стих:

“Завинаги такава си остана -
все търсиш птица, вятър или път.
Очите ти в пространството са взряни,
годините едва ли ще ги спрат.
Река ще си останеш ти до края,
река-немирница - туй знам добре! -
и няма да заглъхнеш в тиха стая,
а все ще търсиш своето море.”
Евтим Евтимов

Разбира се, следват благодарности на семейството, което сама си избрах. Благодаря на приятелите ми, които винаги бяха до мен: на Меги, за всички писма, които си пишехме в края на всяко лято, на Еми, Мина и Ивка, затова че продължаваме да растем заедно. Разбира се, на “Синдикатът”, затова че не спряхте да вярвате в мен, дори когато никой не вярваше, че ще се справя, затова че винаги ме чакате да се върна и ако много се забавя, идвате вие. Благодаря ви, че ме пуснахте да замина през 2013, че плакахте и се смяхте с мен последните поне 15 години. Благодаря ви, че ме търпите, че ме помните и че не ми позволявате никога да забравя, че сте винаги точно до мен. По традиция, всички вас поздравявам с една много шотландска песен с много български текст, която съм сигурна, че е писана за приятелство, поне толкова силно, колкото нашето.

“Налей, ти чашите не брой!
Догоре ги пълни!
Да пием с тебе, друже мой,
за миналите дни!”
За старата любов. Фамилия Тоника

A los que me recibieron con brazos abiertos hace 11 años cuando llegué a Gran Canaria. A Ana, Besay, Nichel, Cristian y Ayoze, por vivir conmigo unos de los mejores años de nuestras vidas y por seguir a mi lado como buenos hermanos. A Fabiola y Isidro, Mari Carmen y Manolo, por asumir el papel de padres locales durante tantos años. A Carmen, por iniciar conmigo esta etapa, por apoyarme, por reirse y llorar conmigo y por vivir juntas tantos momentos de película. A Simone y a Luis, por sujetarme y no dejarme caer, por compartir conmigo este camino y entenderlo en todas sus facetas, porque a veces "solo pasamos 400m en 4 horas". A Ivy, por abrirme tanto las puertas de su casa como de su corazón, por todas esas tardes en el sofá hablando de lo "dura que es la vida". Gracias, por ser mis pilares y por no permitirme nunca pensar que hay algo que no pueda hacer. Esta va por ustedes.

"Y si tengo que morirme
Que me muera en primavera
Pa' poder echar raíces
Y vivir siempre a tu vera
Y si tienes que marcharte
Llévame en una maleta
Yo prometo no pesarte
Tu procura no perderla"
Tragicomedia. Estopa

A Óscar, por ser mi compañero de la carrera más dura de todas, la de la vida. Gracias por permitirme compartir mi tiempo en este mundo contigo. Gracias por prohibirme que deje de creer en mi misma, por levantarme cuando me caía y por vivir mis pequeños logros con más ilusión que yo misma. Gracias por ser y por estar y por permitirme ser parte de tu equipo.

"Mi casa está donde estás tú
Los mismos ojos, la misma luz
Mi casa está donde estás tú
Los mismos clavos, la misma cruz"
Los mismos clavos. Marea

A mis tres directores, gracias, en primer lugar, por la paciencia. Para mí fue un honor poder realizar esta tesis a su lado. A Antonio Fernández, por ser un referente tanto a nivel profesional como personal. Gracias, Toño, por creer en mí en todo momento, por tu comprensión y por tu incesante fe en las capacidades de cada uno de nosotros. Gracias por enseñarme que, siempre y, ante todo, todas las personas somos distintas, únicas y dignas por igual. A Marisa Andrada, gracias por estar siempre y por ser un ejemplo para todo en la vida. Nada de esto sería posible sin ti. Gracias por enseñarme tanto y por dejarme aprender por mí misma. Gracias por apoyarme y estar conmigo en todas las etapas de este viaje, en las buenas y, sobre todo, en las malas. Gracias por toda la ciencia que me dejaste hacer a tu lado, por todos los buenos momentos, por todas las lecciones que aprendimos juntas, y, sobre todo, gracias por ser la persona que eres. A Óscar Quesada, gracias por toda la fe que tuviste en mí, que yo nunca tuve, y, sobre todo, por tu humildad. Gracias por permitirme disfrutar de la patología a tu lado, por apoyarme y animarme siempre, incluso en los momentos más oscuros, por soportar todos mis altibajos, por guiarme y por enseñarme que "hay que hacerlo y punto". Con mucho cariño, les dedico esta rima, que fue la primera poesía que aprendí en español:

Del salón en el ángulo oscuro,
de su dueño tal vez olvidada,
silenciosa y cubierta de polvo
veíase el arpa.
¡Cuánta nota dormía en sus cuerdas,
como el pájaro duerme en las ramas,
esperando la mano de nieve
que sabe arrancarlas!
¡Ay! -pensé-. ¡Cuántas veces el genio
así duerme en el fondo del alma,
y una voz, como Lázaro, espera
que le diga: «Levántate y anda!»
Gustavo Adolfo Becquer

A todo el equipo del IGM (Intituto de Ganadería de Montaña) de León por recibirme, enseñarme, hacerme sentir como en casa y dejarme ser una mas del equipo. A Valentín Pérez, por guiarme desde el cielo. A todos ustedes, gracias por ser una de las principales razones por las que sigo volviendo a León cada vez que tengo la oportunidad.

To the UCD (University College of Dublin) veterinary pathology team, especially to Hanne Jahns, Pamela Kelly, and Joe Cassidy, for the amazing opportunity to learn from you. To the whole team, residents and lab staff, thank you for those three months full of knowledge, laughter, and inspiration. Thanks to Pia for linking our two institutions and for supporting me throughout my whole stay and up until today. And special thanks to Ireland and its people for letting me discover the wonders of this beautiful land. It was a pleasure.

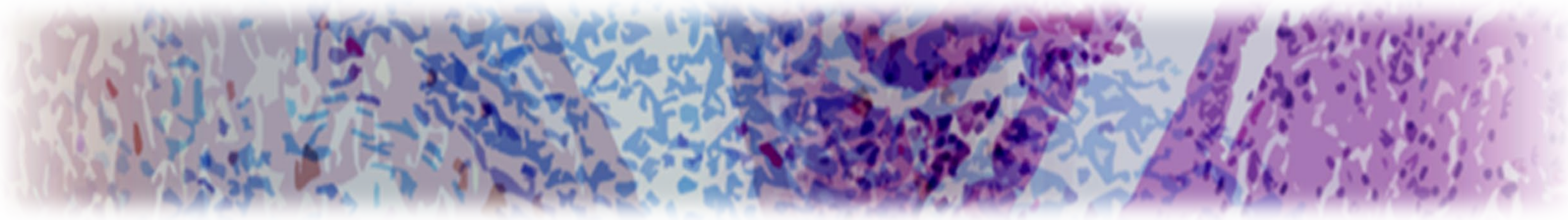
A todos los que han tenido la suerte o la desgracia de ser mis alumnos. Espero haber podido transmitirles lo poco que sé y gracias por haberme enseñado tanto.

Y ahora, un enorme “GRACIAS” a todos mis compañeros del IUSA, tanto docentes, como administrativos, estudiantes, técnicos y “becarios”. A los que me recibieron cuando llegué, a los que me acompañaron durante todos estos años y a los que seguirán por esta senda después de mí. A todos los que forman esta gran familia. Y sin nombrar a nadie y en honor a todas las veces que me oyeron cantar por los pasillos, les dedico esta canción:

“Últimamente pienso en esa gente
En el apuro, siempre presentes
No olvido que os debo una canción
Os debo una canción

Gracias por tanto
Por la risa y el llanto
Gracias por ser parte de mí
Gracias por tanto
Por dulce o por amargo
Gracias por ser parte de mí
Por ser parte de mí”
Te debo una canción. Shinova

Agradecer a las instituciones que han hecho posible este proyecto. Al Instituto Universitario de Sanidad Animal y Seguridad Alimentaria de la ULPGC, al Ministerio de Universidades del Gobierno de España que financió la realización de esta tesis a través del contrato FPU2020. Al Ministerio de Ciencia e Innovación por la financiación del plan nacional PID2021-127687NB-I00, a la Fundación Caixa-Caja Canarias por el proyecto FCC-FC-2019-03, al Gobierno de Canarias, Consejería de Economía, Conocimiento y Empleo; Agencia Canaria de Investigación, Innovación y Sociedad de la Información, destinadas a la realización de proyectos de I+D en las áreas prioritarias de Canarias RIS-3, cofinanciada a través del PO FEDER Canarias 2014 – 2020 por la subvención concedida PROID2020010047 que financiaron las investigaciones desarrolladas en esta tesis. A la organización "Charter100" y la Universidad de Las Palmas de Gran Canaria mediante el programa Erasmus + por financiar las estancias realizadas.



GOBIERNO DE ESPAÑA
MINISTERIO DE CIENCIA E INNOVACIÓN

GOBIERNO DE ESPAÑA
MINISTERIO DE UNIVERSIDADES

Gobierno de Canarias
Consejería de Economía, Industria, Comercio y Conocimiento
Agencia Canaria de Investigación, Innovación y Sociedad de la Información

Unión Europea

Fundación "la Caixa"

CajaCanarias FUNDACIÓN

ULPGC Universidad de Las Palmas de Gran Canaria
IUSA Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

ULPGC Universidad de Las Palmas de Gran Canaria
vet FACULTAD DE VETERINARIA