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ANÁLISE GENÔMICA DE UM SURTO DE TUBERCULOSE BOVINA EM UM SISTEMA MULTI-HOSPEDEIRO ARTIFICIAL: UM CHAMADO À AÇÃO SOBRE A VIDA SELVAGEM NO BRASIL

DAIANE APARECIDA ROSA LIMA

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Genomic analysis of an outbreak of bovine tuberculosis in a man-made multi-host species system: A call for action on wildlife in Brazil

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Universidade Federal de Mato Grosso do Sul, como requisito à obtenção do título de Mestre em Ciências Veterinárias.

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“Em algum lugar, algo incrível está esperando para ser descoberto”
Carl Edward Sagan

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A tuberculose bovina (bTB) é considerada uma das principais doenças que afetam a pecuária brasileira. Bovinos e bubalinos estão inclusos no programa nacional de controle de bTB. Contudo, a ocorrência de casos de bTB na vida selvagem em cativeiro permanece negligenciada e subestimada. Neste estudo, relatamos um surto de 15 anos de bTB em espécies selvagens ameaçadas de extinção em um parque de safári, o qual praticava o comércio interestadual de cervos. Compilamos relatórios do serviço veterinário oficial em uma linha do tempo de eventos, acessamos 21 isolados de *M. bovis* de cervos e dois isolados de lhamas para realizar o sequenciamento de genoma completo para inferir a estrutura populacional, estimativas de datação e eventos de transmissão. Com isso, verificamos que, entre 2003 e 2018, pelo menos 16 animais, de oito espécies, vieram a óbito devido à bTB. No ano de 2015, em três diferentes ocasiões, a população de cervos foi avaliada por testes intradérmicos de tuberculina, sendo positivos ao teste. Dada a falta de uma legislação para a espécie, o serviço veterinário oficial só pôde ordenar o fechamento do parque e o despovoamento de cervos com base no comércio interestadual de animais. A análise genômica indica que múltiplas cepas de *M. bovis* estavam circulando no parque, com pelo menos três introduções diferentes desde a inauguração do parque em 1977, evidenciando sucessivas negligências. Quando comparadas com outros genomas de *M. bovis* de rebanhos bovinos das proximidades, não foram encontrados eventos de transmissão recentes. Ainda assim, sua relação filogenética sugere que linhagens de *M. bovis* foram introduzidas no parque provindas da região, possivelmente por meio da manutenção de gado na propriedade. Por fim, discutimos os fatores socioeconômicos e ambientais que levaram ao surto, ao mesmo tempo que ameaçavam a saúde pública.

Palavras-chave: genoma, *Mycobacterium bovis*, fauna silvestre, tuberculose.

ABSTRACT

LIMA, D.A.R. Genomic analysis of an outbreak of bovine tuberculosis in a man-made multi-host species system: A call for action on wildlife in Brazil 2021. Dissertação de Mestrado – Programa de Pós-Graduação em Ciências Veterinárias. Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, 2022.

Bovine tuberculosis (bTB) is considered one of the main diseases affecting Brazilian livestock. Cattle and buffalo are included in the national bovine tuberculosis control program. However, cases of bTB in Brazilian wildlife in captivity remain neglected and underestimated. In this study, we report a 15-year bTB outbreak in endangered wild species in a safari park, which practiced the interstate deer trade. We compiled reports from the official veterinary service on an event timeline, accessed 21 isolates of *M. bovis* from deer and two isolates from llamas to perform complete genome sequencing to infer population structure, dating estimates and transmission events. With that, we verified that from 2003 to 2018, at least 16 animals, of eight species, died due to bTB. In 2015 on three different occasions, the deer population was evaluated by intradermal tuberculin tests, being positive for the test. Given the lack of legislation for the species, the official veterinary service was only able to order the closure of the park and the depopulation of deer based on the interstate animal trade. Genomic analysis indicates that multiple strains of *M. bovis* were circulating in the park, with at least three different introductions since the park's inauguration in 1977, showing successive neglect. When compared to other *M. bovis* genomes from nearby cattle herds, no recent transmission events were found. Even so, its phylogenetic relationship suggests that *M. bovis* strains were introduced into the park from the region, possibly through the maintenance of cattle on the property. Finally, we discussed the socioeconomic and environmental factors that led to the outbreak, while threatening public health.

Keywords: genome, *Mycobacterium bovis*, wildlife, tuberculosis.

CAPÍTULO 1

1 INTRODUÇÃO

A tuberculose bovina (bTB) é uma doença zoonótica, crônica e debilitante, causada pela bactéria intracelular *Mycobacterium bovis*, pertencente ao complexo *Mycobacterium tuberculosis* (MOL et al., 2016). Esta espécie afeta animais domésticos e silvestres, bem como o homem (KOHL et al., 2018).

A tuberculose bovina causa perdas econômicas relevantes, por afetar a produtividade dos rebanhos e pela eliminação de animais infectados. Constitui também uma zoonose (ABRAHÃO et al., 2005; BENNETT; COOKE, 2006; SA'IDU et al., 2015).

O controle da tuberculose bovina, na maioria dos países, envolve a identificação de animais infectados, por meio de testes intradérmicos, e a eutanásia deles. Por meio da estratégia de teste/abate, alguns países conseguiram erradicar a tuberculose bovina na maior parte dos seus territórios. (KOHL et al., 2018; RIBEIRO et al., 2017). No entanto, a presença de reservatórios silvestres de *M. bovis* tem dificultado a erradicação da enfermidade. Nos Estados Unidos, o veado da cauda branca (*Odocoileus virginianus*) tem importante papel na persistência da tuberculose bovina. Do mesmo modo, no Reino Unido, o texugo (*Meles meles*) tem dificultado a eliminação da enfermidade (CORNER et al., 2011).

No Brasil, há poucos estudos buscando a identificação de reservatórios silvestres da tuberculose bovina. Javalis já foram identificados como potenciais reservatórios (LOPES et al., 2021), no entanto, seu papel epidemiológico ainda não foi completamente elucidado. A infecção por *M. bovis* também tem sido descrita em outras espécies silvestres de vida livre (CORNER et al., 2011; KOHL et al. 2018) ou em cativeiro (LIMA et al., 2021; RIBEIRO et al., 2017; ZIMPEL et al., 2017). Um estudo realizado por Rocha et al. (2011) que relatou um caso de tuberculose bovina em um par de pivas (*Kobus ellipsiprymnus*), em um Zoo Safari no estado de São Paulo. Contudo a fonte de infecção para esses animais mantidos em cativeiro permanece desconhecida (MACIEL et al., 2018).

O uso da epidemiologia molecular tem sido uma ferramenta essencial na

compreensão da estrutura populacional de *M. bovis* e a dinâmica de transmissão em rebanhos e sistemas multi-hospedeiro (GUIMARÃES et al., 2020). Recentemente, o sequenciamento do genoma completo (*whole genome sequencing - WGS*) *de M. bovis* tem possibilitado um entendimento mais preciso e aprofundado desses tópicos. Diversos estudos utilizaram o sequenciamento do genômico de *M. bovis* para avaliar a transmissão de patógenos em rebanhos bovinos e animais selvagens, mostrando a utilidade desta técnica para sistemas de vigilância de bTB (KOHL et al., 2018; LASSERRE et al., 2018). O sequenciamento genômico permite, por exemplo, detectar quantas cepas de *M. bovis* estão circulando em um determinado rebanho, quando essas cepas foram introduzidas e os elos de transmissão entre animais ou fazendas (GUIMARÃES et al., 2020).

Em 1977, um safári privado foi inaugurado no estado do Rio Grande do Sul, Brasil. O safári abrigava diversas espécies de animais, exóticos e nativos, incluindo cervos, capivaras, lhamas, camelos, hipopótamos, diferentes espécies de pássaros, cavalos, primatas não humanos, entre outros. Este sistema multi-hospedeiro artificial apresentou vários desafios sanitários ao longo dos anos, culminando com o seu encerramento, conforme definido por uma ordem judicial em 2013. Entre esses desafios, vários animais morreram de bTB, com relatos que datam desde 2003. Portanto, os objetivos deste estudo foram compilar o histórico de bTB em um parque safári por meio de registros oficiais e avaliar a diversidade das cepas de *M. bovis*, caminhos de transmissão e tempo de introdução de bTB no parque usando dados de sequenciamento genômico.

2 OBJETIVOS

2.1. Objetivo Geral

Realizar a análise genômica de um surto de tuberculose bovina em uma população de animais silvestres em cativeiro.

2.2. Objetivos Específicos

- Sequenciar os genomas de *M. bovis* oriundos de cervídeos e lhamas provenientes de um parque safari;
- caracterizar e analisar a diversidade genética de *M. bovis;*
- identificar os possíveis eventos de transmissão; e
- datar as introduções das cepas de *M. bovis* no parque; e
- realizar uma análise epidemiológica.

3 REVISÃO DE LITERATURA

3.1 Tuberculose e seus hospedeiros

A tuberculose é uma doença infectocontagiosa causada por membros do complexo *Mycobacterium tuberculosi*s (CMT), uma das doenças bacterianas mais prejudiciais que afetam humanos e animais em todo o mundo (KOHL et al., 2018). Propaga-se quando as pessoas que estão doentes com tuberculose expelem bactérias no ar; por exemplo, tossindo. A doença comumente afeta os pulmões (tuberculose pulmonar), mas também pode afetar outros locais (TB extrapulmonar). Considerada pela Organização Mundial da Saúde, uma das 10 principais causas de morte no mundo e a principal causa de morte por um único agente infeccioso, sendo classificada acima de HIV/AIDS (WHO, 2020). Globalmente, estima-se que 10,0 milhões de pessoas foram afetadas pela tuberculose em 2019, com uma estimativa de 1,4 milhões de mortes (WHO, 2020). A tuberculose está intimamente relacionada à realidade socioeconômica, com maior prevalência em países pobres e em desenvolvimento, outro fator que contribui para tais números expressivos é a transmissão zoonótica (bovinos para humanos) (OLEA-POPELKA et al., 2017).

Em 2019, a Organização Mundial de Saúde estimou que ocorreram 140.000 novos casos de TB zoonótica em todo o mundo, devido a *Mycobacterium bovis* (WHO, 2020). A tuberculose bovina (bTB) em humanos é transmitida pela ingestão de leite ou produtos lácteos derivados não pasteurizados e consumo de carne *in natura* proveniente de animais contaminados e, menos recorrente, por contato com animais infectados (SILVA et al., 2018; DE LA RUA-DOMENECH et al., 2006). A bTB, causada por *Mycobacterium bovis*, afeta principalmente bovinos e búfalos, mas possui uma ampla gama de hospedeiros, incluindo espécies silvestres de vida livre e cativas. Sua ocorrência em animais silvestres é um fator importante pois esses animais podem atuar como reservatórios, o que coloca em risco a saúde pública e gera a exposição de espécies ameaçadas de extinção (BUDDLE et al., 2011; O'BRIEN et al., 2011).

Em países desenvolvidos, programas de controle conseguiram erradicar

a bTB, com adesão de testes e abate de animais infectados (CORNER et al., 2011). Contudo, a persistência de bTB em hospedeiros silvestres têm se mostrando um empecilho na erradicação em diversos lugares, como em Michigan, nos Estados Unidos da América (EUA), estado que alcançou o controle da bTB, mas alguns rebanhos continuam infectados devido ao foco da infecção em cervos de cauda branca (*Odocoileus virginianus*) (FINE et al., 2011).

No Brasil, apesar de não terem sido identificados reservatórios selvagens de manutenção para *M. bovis*, há diversos relatos de bTB em animais de zoológico (LIMA et al., 2021; RIBEIRO et al., 2017; ZIMPEL et al., 2017). Entretanto, as principais fontes de infecções que levam aos surtos nacionais de tuberculose bovina e à manutenção da doença ainda são pouco conhecidas. Muitas vezes, o curso da infecção não é esclarecido, dificultando o controle e a interrupção das cadeias de transmissão, gerando resultados insatisfatórios na erradicação da doença em muitos países (KOHL et al., 2018).

Em 2001, o governo brasileiro instituiu o Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose Animal (PNCEBT), estabelecendo estratégias estruturadas de detecção e controle de bTB por meio do teste tuberculínico e vigilância de abatedouros em rebanhos bovinos e bubalinos. Quinze anos depois, a prevalência de bTB em 13 estados foi avaliada por meio do teste cervical comparativo (TCC), representando 75% do rebanho bovino brasileiro (FERREIRA NETO et al. 2016). A situação epidemiológica foi descrita como heterogênea dentro e entre os estados, com prevalência variando de 0 a 2,5% em bovinos e de 0 a 13,9% em rebanhos (FERREIRA NETO et al. 2016). Contudo, os esforços de controle atuais estão principalmente relacionados a espécie bovina, a inserção de espécies selvagens em programas de controle e erradicação, seria de suma importância, uma vez que pouco se sabe sobre a capacidade de transmissão, virulência e persistência das estirpes nacionais de *M. bovis* e seus determinantes genéticos.

3.1.2 Adaptabilidade bacteriana ao hospedeiro

Patógenos pertencentes ao CMT possuem diferentes fenótipos de patogenicidade e adaptação ao hospedeiro, agindo conforme a espécie animal

infectada (COUSINS et al., 1994, COSCOLLA et al., 2013; GAGNEUX et al., 2014). As espécies de micobactérias relacionadas aos animais são nomeadas conforme o hospedeiro em que foram primeiramente identificadas ou em que ocorram com maior frequência. As espécies *M. tuberculosis* e *M. bovis* possuem maior importância para saúde pública e a pecuária, estando amplamente distribuídas pelo mundo provocando doenças em seres humanos e podendo afetar animais em eventos de *spillover.* Algumas espécies animais infectadas por *M. bovis* podem ser consideradas reservatórios, e outras como hospedeiros finais (*dead-end*). As causas determinantes dessa variável permanecem desconhecidos (BEHR; GORDON, 2015).

Outras espécies do CMT são: *M. caprae* em ruminantes domésticos da Europa, *M. pinnipedii* em mamíferos marinhos na Oceania*, M. microtti* em roedores na Grã-Bretanha, *M. orygis* em antílopes geralmente no continente Africano, *M. suricattae* em suricatos, *M. mungi* em mangustos, “dassie bacillus” em hírax e por fim “chimpazee bacillus” (SMITH et al., 2006; ALEXANDER et al., 2010; COSCOLLA et al., 2013; COSCOLLA; GAGNEUX, 2014; GALAGAN, 2014; RODRIGUEZ-CAMPOS et al., 2014; DIPPENAAR et al., 2015).

3.1.3 Patogenia e sinais clínicos

A infecção causada por *M. bovis* é caracterizada pela formação de lesões nodulares granulomatosas, denominados de tubérculos. A formação dos tubérculos se dá, quando o bacilo é capturado e fagocitado por macrófagos e células dendríticas, e quando não ocorre a lise dos bacilos, estes se multiplicam dentro dos macrófagos até destruí-los (ANDREWS et al., 2008; VERMA et al., 2014). Algumas semanas após infecção, cessa-se a multiplicação dos bacilos devido à resposta imune mediada por células e pela reação de hipersensibilidade tardia, e com o acúmulo de células inflamatórias desencadeia-se a formação do granuloma (ANDREWS et al., 2008; ABOUKHASSIB et al., 2016). As lesões macroscópicas são caracterizadas por linfonodomegalia com granulomas formados por exsudato caseoso seco e mineralizado, de coloração amarelo-esbranquiçado a verde-esbranquiçado. Aspecto purulento, com cápsula fibrosa, com ou sem necrose de caseificação no centro da lesão ou calcificação também podem ser observadas. (ZACHARY; MCGAVIN, 2013).

Lesões tuberculosas podem ser observadas em qualquer tecido do animal, mas frequentemente são encontradas nos pulmões, fígado, baço, linfonodos traqueobrônquicos, mediastinais, retrofaríngeos e brônquicos. Geralmente, inicia-se na junção bronquíolo-alveolar com propagação para outros tecidos, podendo progredir, estabilizar ou regredir. A disseminação para outros tecidos é determinada pela carga infectante, virulência da micobactéria e resistência do hospedeiro (ZACHARY; MCGAVIN, 2013; SOUZA et al., 2014; VERMA et al., 2014).

Considerada uma doença de evolução lenta, a fase inicial da bTB comumente é assintomática e mesmo em fases avançadas, os sinais clínicos podem se manifestar após meses ou anos (OIE, 2018). Quando presentes, os sinais clínicos podem variar de tosse crônica, emagrecimento progressivo, dispneia, hiperplasia de linfonodos, mastite, estado febril intermitente, infertilidade ou aborto e em alguns casos diarreia (ANDREWS et al., 2008; QUINN et al., 2005; VERMA et al., 2014; OIE, 2018).

3.2 Ferramentas moleculares para avaliação epidemiológica de *Mycobacterium bovis*

Metodologias como espoligotipagem (*spolityping*) e número variável de repetições em sequência *(VNTR - Variable Number Tandem Repeat)* e Unidades Repetitivas Micobacterianas (MIRU) (MIRU-VNTR) estão sendo amplamente utilizadas como formas de avaliar a distribuição epidemiológica de *M. bovis.* Tais métodos são realizados por meio da técnica da PCR (Reação em Cadeia da Polimerase), comumente de forma complementar, e usadas em investigações de surtos da tuberculose (ZHANG et al., 2010).

A espoligotipagem, é um método de genotipagem molecular baseado em PCR, no qual ocorre a amplificação do locus cromossomal de micobactérias. Essa região cromossomal possui inúmeras e pequenas regiões de repetição direta (DR, do inglês, *Direct Repeat*), e cada DR é intercalada com sequências de espaçadores únicos e não repetitivos que estão presentes apenas em bactérias do CMT. Regiões DRs quando comparadas a outras cepas isoladas do CMT, apresentam uma sequência de espaçadores, com deleções e inserções.

(KAMERBEEK et al., 1997). Com o surgimento do sequenciamento genômico, a espoligotipagem pode ser alternativamente realizada com as *reads* (resultado obtido após o sequenciamento de genomas, corresponde a uma pequena sequência de poucos pares de base) do sequenciamento genômico, com genomas completos ou *drafts* (genoma incompleto) através dos programas SpolPred e SpoTyping (COLL et al., 2012; XIA; TEO; ONG, 2016). O uso de espoligotipagem isoladamente, não é aconselhável para epidemiologia molecular, pois homoplasias podem resultar em um mesmo padrão de espoligotipo, fazendo com que seja encontrado em diferentes linhagens de *M. bovis* (SMITH, 2012).

Já em relação ao MIRU-VNTR, que foi primeiramente padronizado para *M. tuberculosis*, tem como objetivo analisar diferentes loci ao longo do genoma e é baseado em 24 regiões que resultam em diferentes tamanhos em decorrência do padrão de repetições em cada locus (SUPPLY et al., 2006). Desta forma, o uso dessas técnicas em conjunto torna a análise epidemiológica mais confiável (SUPPLY et al., 2006).

3.3 Evolução do complexo *Mycobacterium tuberculosis* (CMT)

Sugere-se que os membros do complexo *M. tuberculosis* sofreram um gargalo evolutivo no momento da especiação, há cerca de 15.000-20.000 anos atrás (SREEVATSAN et al., 1997). Micobactérias pertencentes ao CMT são caracterizadas por 99,95% de identidade de nucleotídeos em regiões alinhadas e sequências de rRNA 16S idênticas, que evoluíram clonalmente de um ancestral semelhante a *M. tuberculosis* por meio de deleções e inserções sucessivas de DNA, resultando na especiação de *Mycobacterium* (BROSCH et al., 2002). Apesar de sua grande similaridade genética, os membros do CMT diferem nas espécies devido a variações genotípicas específicas, como polimorfismos de nucleotídeo único (SNPs) e deleção de regiões de diferença (RDs), em termos de tropismos do hospedeiro e patogenicidade (BROSCH et al., 2002; GALAN, 2014).

Já se considerou que *Mycobacterium canettii* pertencia ao CMT, porém este patógeno está associado a infecções em humanos, e nunca foi descrito em animais (ABOUBAKER OSMAN et al., 2015). De acordo com Supply et al.

(2013), essa espécie não é agrupada no complexo por possuir características que não são descritas nos membros do CMT, como: um genoma maior e indícios de sofrer recombinações e transferência horizontal de genes (HGT). Desta forma, sugere-se que *M. tuberculosis* é o microrganismo mais próximo do ancestral comum (GALAGAN, 2014; RODRÍGUEZ-CAMPOS et al., 2014) que deu origem ao MTBC e a *M. canettii*.

3.4. Estrutura genômica de *Mycobacterium bovis*

Garnier et al. (2003) sequenciaram o genoma completo da cepa virulenta *M. bovis* AF2122/97, obtida, em 1997, a partir do isolamento de lesões nos pulmões e linfonodos bronco-mediastinais de uma vaca na Grã-Bretanha em 1997 (GORDAN et al., 1999). O genoma desta cepa de *M. bovis* é composto por 4.345.492 pb (pares de base), organizado em um único cromossomo circular composto por 65,63% de guanina e citosina. Formado de 3.952 genes que codificam proteínas, que inclui um profago e 42 elementos de inserção (*insertion sequence* - IS). O genoma de *M. bovis* AF2122/97 possui 99,95% de semelhança no alinhamento com *M. tuberculosis* demonstrando colinearidade e nenhuma evidência de grandes translocações, duplicações ou inversões, contudo a exclusão da informação genética reduziu o tamanho do seu genoma (GARNIER et al., 2003). Quando comparado a *M. leprae,* revela uma série de perdas genéticas que implicam na expressão gênica diferencial, podendo ser considerada a chave para os tropismos do hospedeiro de bacilos humanos e bovinos. A sequência do genoma, permite uma visão importante na evolução, sendo as deleções no DNA são uma das principais contribuições dessas diferenças (GARNIER et al., 2003; ZIMPEL et al., 2017). Os componentes da parede celular e as proteínas secretadas demonstram maior variabilidade, indicando um papel importante nas interações hospedeiro-bacilo ou na evasão imunológica. Além disso, não existem genes exclusivos para *M. bovis* (GARNIER et al., 2003), desta forma, demonstrando a importância de se identificar as espécies dos isolados do CMT para estudos epidemiológicos e saúde pública.

3.4.1 Regiões de diferenciação

Brosch et al. (2002) apresentaram um novo cenário evolutivo para os membros do CMT, revelando 20 regiões de diferença no genoma dessas espécies. Catorze dessas denominadas RD1-RD14 com variação de tamanho entre 2 a 12,7 kb e se mostraram ausentes no bacilo Calmette-Guérin Pasteur quando comparadas com a cepa H37Rv de *M. tuberculosis*. Seis delas denominadas RvD1-RvD5 e TbD1 se mostraram ausentes na cepa H37Rv quando comparadas com outros membros do complexo.

Com a descoberta dessas regiões de diferença no genoma do CMT, o diagnóstico molecular e a diferenciação de espécies são considerados padrão-ouro para diferenciar os membros desse complexo. Com isso, *M. bovis* pôde ser diferenciado com precisão em relação a outros membros do CMT pelas regiões deletadas RD4 e RD9, e de *M. bovis* BCG pela ausência de RD1BCG (que é deletada nas cepas de BCG). Na análise de distribuição de RDs nas cepas de *M. bovis* distinguiu-se cinco principais grupos, que se caracterizaram pelas deleções de RD4, RD5, RD7, RD8, RD9, RD10, RD12 e RD13, com exceção dos profagos. Entretanto, as regiões RvD foram altamente conservadas e TbD1 estava presente em todas os isolados. Os resultados apresentados mostram, que essa espécie do CMT seria o último membro de uma linhagem separada representada por *M. africanum* (RD9) e *M. microti* (RD7, RD8, RD9, RD10) e que se ramificaram do progenitor de *M. tuberculosis*, representante único da RD9. A perda sucessiva de DNA pode ter sido um fator contribuinte na expansão clonal e na origem de patógenos em novos hospedeiros (BROSCH et al., 2002). O método de PCR com base nas regiões de diferenciação (RD1, RD1mic, RD2seal, RD4, RD9 e RD12), foi desenvolvido para diferenciar membros do CMT (*M. tuberculosis, M. africanum, M. microti, M. pinnipedii, M. caprae, M. bovis* e *M. bovis* BCG). Com isso, cepas de *M. bovis* podem se distinguidas de outras espécies do CMT pela ausência de RD9 e RD4, já *M. bovis* BCG se diferencia pela ausência de RD1BCG (WARREN et al., 2006). Em um recente estudo baseado em análises genômicas pode-se confirmar que todos os membros do CMT conhecidos como adaptados a animais possuem um ancestral comum no

ponto de ramificação que é caracterizado pelas deleções de RD7, RD8, RD9 e RD10 (BRITES et al., 2018).

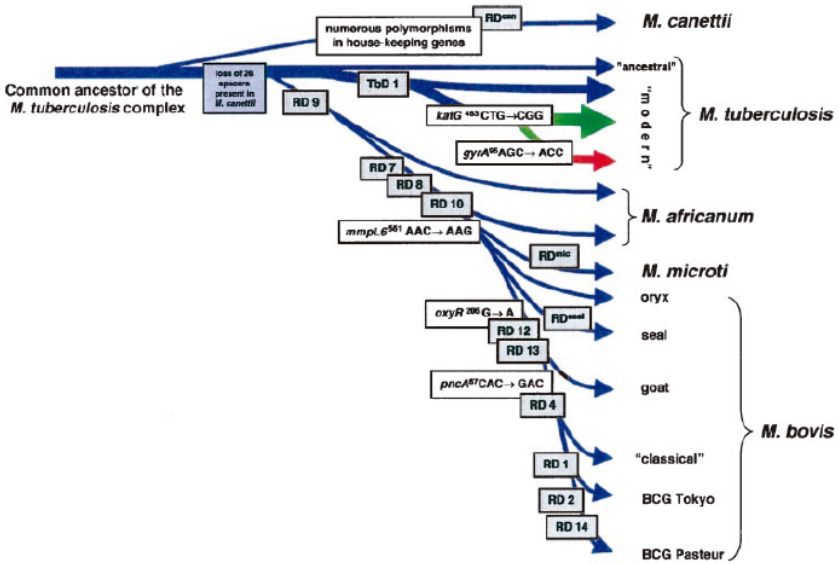

Figura 1. Escala evolutiva dos membros do complexo *Mycobacterium tuberculosis.* Fonte: (BROSCH et al., 2002).

3.4.2 Complexos Clonais

A estrutura populacional de *M. bovis* pode ser definida como irregular e não uniforme, que se constitui em uma série de pequenos e grandes complexos clonais (SMITH et al., 2003), sendo complexos clonais grupos de cepas descendentes de uma única célula que foi o ancestral comum mais recente, e que possui todas as características oriundas do ancestral comum mais recente (SMITH et al., 2011). Ainda que a tuberculose bovina tenha sido relatada em todos os continentes, é sabido que este patógeno se originou em um só lugar, de uma só vez, e a partir disto tem sido distribuído mundialmente (SMITH et al., 2011).

Determinados complexos clonais de *M. bovis* foram caracterizados por deleções específicas. Dois desses complexos estão geograficamente localizados na África. O complexo clonal África1 (Af1) (MULLER et al., 2009) foi identificado nas regiões de Camarões, Nigéria, Mali e Chade, sendo caracterizado devido à deleção cromossômica específica (RDAf1) e pela

ausência do espaçador 30 na técnica de *spoligotyping.* Já o complexo clonal África2 (Af2) (BERG et al., 2011) foi observado com maior frequência na Uganda, Burundi, Tanzânia e Etiópia, definido pela deleção cromossômica RDAf2 e ausência dos espaçadores 3-7 no padrão de *spoligotyping.* (SMITH et al. 2011).

Identificado por Smith et al. (2011), o terceiro complexo clonal, possui distribuição global e é nomeado Europa 1 (Eu1), determinado pela exclusão da região cromossômica RDEu1, perda de espaçador 11 no padrão *spoligotyping*, apesar desta característica não ser obrigatoriamente específica para este complexo clonal. Países como: República da Irlanda, Reino Unido, Estados Unidos, África do Sul, Nova Zelândia, Austrália, Canadá, Argentina, Chile, Equador, México, Coréia e do Cazaquistão, apresentaram maior incidência de cepas identificadas como complexo clonal Europa1. Em países como Brasil, Franca, Espanha, Portugal e Irã, foi pouco observado, havendo a hipótese de que a distribuição desse complexo clonal ocorreu por meio de bovinos infectados do Reino Unido e antigos parceiros comerciais (SMITH et al., 2011).

Diferentemente dos já mencionados, o quarto complexo clonal Europa 2 (Eu2) (RODRIGUEZ-CAMPOS et al., 2012) é oriundo da Península Ibérica, onde são encontrados com maior assiduidade, definido pela ausência do espaçador 21 associado a um SNP no gene *guaA*. Cepas de *M. bovis* de membros do Eu2 são observadas também em países como França, Itália e Ilhas Britânicas, mas em uma menor frequência. A mutação no gene *guaA* não foi observada nas cepas de referência dos três complexos clonais anteriormente descritos (Af1, Af2 e Eu1), sugerindo que o Eu2 compõe um quarto complexo clonal de importância global (RODRIGUEZ-CAMPOS et al., 2012). Um estudo recente de genomas de *M. bovis* de vários países mostrou que os complexos clonais historicamente observados (isto é, africanos 1 e 2, europeus 1 e 2) (RODRIGUEZ-CAMPOS et al., 2012; SMITH et al., 2011) não representam toda a diversidade genética deste patógeno, e a existência de pelo menos quatro linhagens (Lb1-Lb4) e três “grupos desconhecidos” de *M. bovis* foram sugeridos (ZIMPEL et al., 2020).

Estudos realizados no Brasil, demonstraram que apenas dois complexos clonais estão presentes, sendo eles complexo clonal Eu1 e Eu2 (CARNEIRO et al., 2021; CONCEIÇÃO et al., 2020; PATANÉ et al., 2017; ZIMPEL et al., 2020).

SP38 e SP35, foram as primeiras cepas de *M. bovis*, reportadas como representantes do complexo clonal Eu2 no país (ZIMPEL et al., 2017). Têm sido relatados por alguns estudos, que genomas de *M. bovis* não possuem nenhum marcador genético para complexo clonal, sugerindo a hipótese de que os complexos clonais não expõem toda a diversidade de linhagens de *M. bovis* (ZIMPEL et al., 2017; GHEBREMARIAM et al., 2018; CONCEIÇÃO et al., 2020 LOISEAU et al., 2020). Com isto, sugere-se a existência de quatro linhagens globais de *M. bovis*, Lb1 e Lb2 (África e Europa), Lb3 (Américas) e Lb4 (distribuição global), não sendo totalmente representadas por complexos clonais, e espalhadas conforme sua localização geográfica ao invés de espécies hospedeiras. Linhagens Lb2 (Af1) e Lb4 (Eu1) possuem marcadores de complexos clonais estáveis, porém linhagens Lb1 e Lb3 são identificados com maior precisão por meio de sequenciamento genômico e conjunto de SNPs (ZIMPEL et al., 2020). Loiseau et al. (2020) descrevem outros novos oito clados, não previamente caracterizados. De maneira geral, estudos baseados em complexos clonais de *M. bovis* contribuem na interpretação de dados genotípicos coletados por epidemiologistas moleculares, com suma importância na criação de hipóteses para investigar a patogenicidade e a disseminação da doença (SMITH, 2012).

3.4.3 Sequenciamento genômico

O uso de tipagem molecular em isolados de *M. bovis* baseado em elementos genéticos repetidos tem sido de grande importância em estudos epidemiológicos e no controle de bTB (COUSINS et al., 1998; SKUCE; NEILL, 2001). Contudo, métodos de genotipagem apresentam limitações em distinguir eventos, dentro de grupos e na mensuração da disseminação de TB, apesar de serem úteis para identificar agrupamentos locais (TREWBY et al., 2016). O surgimento do sequenciamento completo do genoma de *M. bovis*, possibilitou uma nova perspectiva de genotipagem (GARNIER et al., 2003). Desta forma, as metodologias de sequenciamento genômico de nova geração trouxeram avanços nas investigações de surtos de doenças (WALKER et al., 2013), possibilitando a análise de transmissão da doença em diferentes escalas, desde nível individual a rebanho, permitindo a inferência da estrutura de contatos entre

as populações, por meio das observações de mutações no genoma bacteriano (KAO et al., 2016; PATANÉ et al., 2017). Esta técnica tem sido utilizada com sucesso para direcionar focos e estudos epidemiológicos no Reino Unido (TREWBY et al., 2016) e nos Estados Unidos (ORLOSKI et al., 2018), e considerada a ferramenta molecular que forneceu melhor resolução, quando comparada a outros métodos de genotipagem (PRICE-CARTER et al., 2018).

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ORIGINAL ARTICLE

Genomic analysis of an outbreak of bovine tuberculosis in a man-made multi-host species system: A call for action on wildlife in Brazil

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Abstract

We report on a 15-year-long outbreak of bovine tuberculosis (bTB) in wildlife from a Brazilian safari park. A timeline of diagnostic events and whole-genome sequencing (WGS) of 21 *Mycobacterium bovis* isolates from deer and llamas were analyzed. Accordingly, from 2003 to 2018, at least 16 animals, from eight species, died due to TB, which is likely an underestimated number. In three occasions since 2013, the deer presented positive tuberculin tests, leading to the park closure and culling of all deer. WGS indicated that multiple *M. bovis* strains were circulating, with at least three founding introductions since the park inauguration in 1977. Using a previously sequenced dataset of 71 *M. bovis* genomes from cattle, we found no recent transmission events between nearby farms and the park based on WGS. Lastly, by discussing socio-economic and environmental factors escaping current regulatory gaps that were determinant of this outbreak, we pledge for the development of a plan to report and control bTB in wildlife in Brazil.

KEYWORDS
bovine tuberculosis, genomics, *Mycobacterium bovis*, public health policies, wildlife

1 | INTRODUCTION

Bovine tuberculosis (bTB) is an OIE (World Organisation for Animal Health) notifiable disease with major impact on livestock and wildlife (OIE, 2011). The disease is present in most countries, but with variable prevalence (End TB Strategy & Cousins, 2018). While certain developed nations have significantly reduced or eradicated the disease, developing countries with absent or inefficient control programs struggle to contain it, leading to a significant toll on livestock producers and/or devastating effects on wildlife (Ayele et al., 2004; Ferreira Neto, 2019; O'Reilly & Daborn, 1995). bTB can also be transmitted to humans through close contact with infected animals or the consumption of unpasteurized milk (Olea-Popelka et al., 2017). It is estimated that 147,000 new cases and 12,500 deaths occur due to zoonotic TB every year (WHO, FAO, & OIE, 2017). Recently, the OIE, WHO (World Health Organization), The Union, and FAO (Food and Agriculture Organization) launched the roadmap for zoonotic TB, urging stakeholders to apply the One Health approach to tackle zoonotic TB and contribute to achieve the sustainable development goal of ending the global TB epidemic by 2030 (WHO, FAO & OIE, 2017).

Brazil is the second largest beef producer in the world and the number one exporter. In 2001, the Brazilian government launched the National Program for the Control and Eradication of Animal Brucellosis and Tuberculosis (PNCEBT), setting structured strategies of bTB detection and control through tuberculin skin testing (TST) and slaughterhouse and trade surveillance in cattle and buffalo herds. Fifteen years later, the prevalence of bTB in 13 states was assessed through TST, representing 75% of the Brazilian bovine herd (Ferreira Neto et al., 2016). The epidemiologic status was described as heterogeneous within and among states, with the prevalence varying from 0% to 2.5% in cattle and from 0% to 13.9% in herds (Ferreira Neto et al., 2016). In contrast to few other countries, a wildlife reservoir of *Mycobacterium bovis*, the main causative pathogen of bTB, has not been identified in Brazil until now, hence current bTB control efforts are mostly concerned with cattle transit and introduction of infected cattle into uninfected herds. In places such as New Zealand, United Kingdom, Michigan (USA) and others, the presence of wildlife reservoirs is an impediment to the eradication of bTB, and it is thus important to maintain surveillance for the emergence of potential reservoirs in Brazil.

Molecular epidemiology has been a resourceful tool to understand *M. bovis* populational structure and transmission dynamics in livestock and multi-host systems (Guimaraes & Zimpel, 2020; Kao et al., 2016). More recently, whole genome sequencing (WGS) of *M. bovis* has allowed a more precise and in depth understanding of these topics. A recent study of *M. bovis* genomes from multiple countries showed that the historically used clonal complexes (i.e., European 1 and 2, African 1 and 2) (Berg et al., 2011; Müller et al., 2009; Rodriguez-Campos et al., 2012; Smith et al., 2011) do not represent the whole genetic diversity of this pathogen, and the existence of at least four lineages (Lb1–Lb4) and three 'unknown groups' of *M. bovis* was suggested (Zimpel et al., 2020). On the other hand, numerous other studies have used WGS of *M. bovis* to evaluate pathogen transmission in cattle herds and wildlife (reviewed by Guimaraes & Zimpel, 2020), showing the usefulness of this technique for bTB surveillance systems. WGS allows, for instance, to detect the number of *M. bovis* sequence types circulating in a particular herd, when these strains were introduced, and the transmission links among animals or farms (Guimaraes & Zimpel, 2020; Kao et al., 2016).

In Brazil, despite the rare bTB detection in free-ranging wildlife (Maciel et al., 2018), reports of *M. bovis* infecting captive wild animals are frequent (Ikuta et al., 2018; Murakami, Monego, Ho, Gibson, De Castro Vilani et al., 2012; Rocha et al., 2011; Zimpel et al., 2017). The source of infection to these zoo animals, however, remains elusive. In 1977, a privately owned safari park was inaugurated in the state of Rio Grande do Sul, Southern Brazil for p

ublic entertainment and environmental education. This safari comprised an area of 320 acres and harboured many different species of animals, both exotic and native, including deer, capybaras, llamas, camels, hippopotamus, different bird species, horses, non-human primates, among others. This man-made multi-host system posed various sanitary challenges over the years, culminating with its closure as defined by a court order in 2013. Among these challenges, several animals died of TB, with reports dating back to 2003. Therefore, the aims of this study were to compile the TB history of this safari park through official records and to evaluate *M. bovis* strain diversity, time of bTB introduction in the park, and path of transmission using WGS data. To achieve these objectives, we sequenced *M. bovis* genomes of 19 deer culled in 2018 and added other two *M. bovis* genomes obtained from llamas in 2012, sequenced in a previous study (Zimpel et al., 2020). We also assessed 71 previously sequenced *M. bovis* genomes (Rodrigues et al., 2021) from nearby cattle farms to verify recent transmission events from the local area to the safari park. The events that took place in the safari park described in this study highlight many regulatory gaps of the current bTB control and animal welfare legislations in the country. While we were only able to access the data presented herein many years after these events occurred, we take this opportunity to launch a call for action for the control of TB in captive wild animals in Brazil.

2 | MATERIALS AND METHODS

2.1 | Study site, official records and deer sampling

This study was carried out in a privately owned safari park located in the state of Rio Grande do Sul, Southern Brazil (Figure 1). To understand and describe the history of bTB in this park, we assessed records of the official state veterinary service that was used as a basis to close the park by court order in 2013 due to sanitary problems. In this official record, necropsy and laboratory reports from different public universities and private diagnostic laboratories dating back from 2003 were available. We then interviewed a previous employee of the park to confirm details about the park history, leading to a compilation of all documents and information to produce a chronological series of events that occurred at the park over the years.

We had access to tissue samples of deer that were euthanized after 16 of them (out of 51 tested) presented positive results in comparative

FIGURE 1 Location of the safari park (red drop) and the cities of the cattle farms are represented by different colours on the map of the Rio Grande do Sul state, Southern Brazil. The safari park is located in the vicinity of Porto Alegre, the capital city of Rio Grande do Sul. Pink: cattle herd from Não-Me-Toque; Midnight Green: Arroio do Tigre; Zomp: Teutônia; Green: Charqueadas; Coral Pink: Estrela; Orange: Fazenda Vila Nova; Cyan: Bom Retiro do Sul; Purple: Eldorado do Sul; Black: Camaquã

cervical tests using *M. bovis* and *M. avium* PPD (purified protein derivative). Briefly, TST was carried out in 51 deer by the official veterinary service of the state, following the United States Department of Agriculture (USDA) protocol, on three different occasions from 2013 to 2015. A total of 16 deer tested positive, indicating the presence of bTB in the population. After judicial procedures, in 2018, all 281 deer kept in the park were culled. Retropharyngeal, submandibular and mesenteric lymph nodes were conveniently collected from 21 deer (*Cervus unicolor*, *Cervus elaphus* and *Dama dama*) with lesions suggestive of tuberculosis. From these, 19 isolates of *M. bovis* were obtained as previously described (Lima et al., 2021). Unfortunately, the name of the host species of each sample was not recorded at the time of sampling.

2.2 | DNA extraction of *M. bovis* from deer

DNA was extracted from the 19 *M. bovis* isolates from deer following an adapted protocol (Van Embden et al., 1993) at the Animal Immunology Laboratory, a biosafety level 3 facility located in the Department of Animal Health, Embrapa Beef Cattle, Campo Grande, Mato Grosso do Sul, Brazil.

2.3 | Whole genome sequencing of *M. bovis* from deer

DNA samples of *M. bovis* isolates from deer were sent to Oswaldo Cruz Institute (Fiocruz), Manguinhos, Rio de Janeiro, Brazil, for WGS. The concentration and quality of DNA were measured using Qubit fluorometer (Invitrogen, California, USA) and Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Paired-end genomic libraries were constructed using Nextera DNA Flex Library Prep kit (Illumina, California, USA), according to the manufacturer's instructions. Genomic libraries were then sequenced in HiSeq 2500 platform (Illumina) using HiSeq Rapid SBS Kit V2 (200 cycles). Illumina sequencing reads were made available in the Sequence Read Archive (SRA), NCBI (Table S1).

2.4 | *Mycobacterium bovis* genomes from llamas

Mycobacterium bovis was isolated from two llamas of the same safari park in 2012 as part of an unrelated study (Ikuta, 2016) and their genomes sequenced in 2019 (Zimpel et al., 2020) (Illumina, paired-end, 100 bp – llama #1 and 250 bp – llama #2). Both genomes were included in this study (Table S1) and compared with *M. bovis* genomes obtained from the deer.

2.5 | *Mycobacterium bovis* genomes from cattle herds in Rio Grande do Sul

For comparison purposes, 71 sequencing reads (Illumina, paired-end, 100 bp) obtained from 13 cattle herds in Rio Grande do Sul, the same state of the safari park, were included in the analysis. These *M. bovis* isolates were obtained and sequenced in an unrelated study

(Rodrigues et al., 2021). Figure 1 indicates the location of the safari park in relation to these cattle herds.

2.6 | Quality control and reads mapping

Sequencing files were trimmed and the adapters were removed using Trimmomatic 0.39 (sliding window 5:20) (Bolger et al., 2014). The quality of the trimmed reads was evaluated using FastQC (Simon, 2010). Genomes had to meet the following quality criteria to be included in the study: GC content of ~65% without multiple or anomalous peaks, coverage of at least 15×, median read length ≥ 70 bp and absence of low-quality sequences. Reads were mapped using Burrows-Wheeler Aligner 0.7.17 (BWA-MEM) (Li & Durbin, 2010) against *Mycobacterium tuberculosis* H37Rv to check that genomes had a sequencing coverage of at least 95% and for the identification of regions of difference (RDs). Accordingly, the resulting bam files were evaluated for the absence of RD4 and presence of RD1 (*M. bovis*-specific and to rule out bacillus Calmette-Guérin, respectively) using an algorithm previously described (Zimpel et al., 2020). Quality-approved reads were then mapped against *M. bovis* AF2122/97 using BWA-MEM, and Picard v2.18.23 (https://github.com/broadinstitute/picard) was used to remove duplicates from resulting files.

2.7 | Variant calling

Single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) were called as previously described (Zimpel et al., 2020) using samtools mpileup (Li, 2011) and VarScan mpileup2cns (Koboldt et al., 2012) with parameters of minimum read depth of seven, mapping quality and minimum base quality of 20 and strand bias filter on, followed by annotation using SnpEff (Cingolani et al., 2012). INDELs as well as SNPs from repetitive regions (PE/PPE, transposases, integrases, maturase, phage and repetitive family 13E12 genes) were removed from the analysis (Zimpel et al., 2020). Genomes were excluded if the number of heterogeneous SNPs exceeded 15% of the total number of detected SNPs in a genome (Zimpel et al., 2020).

2.8 | Phylogenetic reconstruction and principal component analysis of *M. bovis* from the safari park

A core-SNP matrix of *M. bovis* genomes from the safari was generated using a previously described algorithm (Zimpel et al., 2020) and subjected to ascertainment bias correction (ASC) using IQ-Tree -fconst (Nguyen et al., 2015). The ASC-corrected matrix was used in the ModelFinder program (Kalyaanamoorthy et al., 2017) to select the best substitution model according to Bayesian Information Criterion (BIC). The best model, TVM+F+R10, was fixed to reconstruct the phylogeny using maximum likelihood (ML) algorithm with 1,000 UFBoot pseudo-replicates (Hoang et al., 2017). *Mycobacterium bovis* AF2122/97 was included in the analysis and five representative genomes of *Mycobacterium caprae* were used as outgroup (ERR1462591, ERR1462625, ERR1462617, ERR1462581, ERR1462585).

To evaluate clustering among *M. bovis* isolates, a principal component analysis (PCA) was constructed based on the SNP matrix. The function princomp was used in R software 3.5.0 to generate two- and three-dimensional PCA graphs.

2.9 | Clonal complexes and spoligotyping

The clonal complexes African 1 and 2, and European 1 (Berg et al., 2011; Michel et al., 2010; Smith et al., 2011) were identified using the bam files generated from mapping the reads against *M. tuberculosis* H37Rv as previously described (Zimpel et al., 2020). The SNP in the *guaA* gene, predictive of clonal complex European 2, was investigated in the same bam files by checking the position 3,813,236 (Rodriguez-Campos et al., 2012). Spoligotype patterns were predicted with SpoTyping software (Xia et al., 2016) using the fastq files. The SB numbers were retrieved from the online database (*M. bovis* Spoligotype Database – mbovis.org).

2.10 | Dating estimates

Dating analyses were performed in BEAST v1.10.4 (Suchard et al., 2018). The phylogenetic tree was estimated altogether with time inference of nodes. Isolation dates were used for tip-dating (Rieux & Balloux, 2016), with a relaxed clock model (uncorrelated lognormal rates across branches). The prior for the clock rate (parameter *ucln.mean*) was set as (1e-9; 2e-7) substitutions/site/branch/year (s/s/b/y), a conservative range encompassing values from previous studies (Bos et al., 2014; Eldholm et al., 2015; Kay et al., 2015; Lillebaek et al., 2016; Menardo et al., 2019; Pepperell et al., 2013). Markov Chain Monte Carlo (MCMC) chains were run to avoid convergence to local optima, each run for 10^7 generations, or until convergence of parameters and effective sample sizes (ESS) > = 200 (checked in Tracer v1.7.1 [Rambaut et al., 2018]). Burnin trees were discarded, and the remaining ones were annotated using TreeAnnotator (Rambaut & Drummond, 2010), generating a maximum clade credibility tree with median heights. Intervals for ages are reported for each node of the phylogenetic tree, as the (minimum between two runs and maximum between two runs), to be conservative. The same five genomes of *M. caprae* described above were used as outgroup.

2.11 | Pairwise SNP distance and minimum spanning tree

The core-SNP matrix using *M. bovis* genomes from the safari park and from cattle herds was used to construct a matrix of SNP distances between genomes and a minimum spanning tree using PHYLOViZ 2.0 (Francisco et al., 2012) with default parameters.

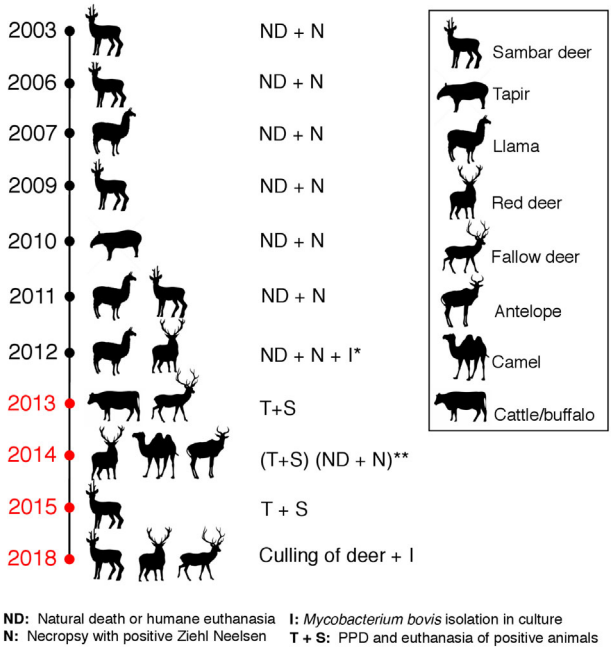

FIGURE 2 Timeline of the tuberculosis outbreak in the safari park according to data collection of government authorities from the state of Rio Grande do Sul, Brazil. Years in red represent zoo closure (for visitors), following a court order in 2013. **Mycobacterium bovis* isolation performed in two llamas, and positive PCR in a third animal. Isolation was not performed in the red deer. ** T+ S in deer, ND + N in a camel and an antelope. PPD: Purified protein derivative of *M. bovis* – indicates that animals were subjected to tuberculin test (comparative cervical test)

3 | RESULTS

3.1 | bTB history in the park

From the investigation of official records, we delineated a timeline of all TB cases that culminated in animal deaths from 2003 to 2018 in the safari park (Figure 2). In brief, from 2003 to 2015, at least 16 animals, from eight different species, died or were humanely euthanized due to TB. Except for two llamas in 2012, TB was only diagnosed based on necropsy findings and Ziehl–Neelsen staining of tissue samples (Figure 2). There was an overall increase in sanitary problems in the park starting around 2005, which coincides with management changes (personal communication of a previous employee). These changes resulted in overpopulation (particularly of deer) and poor feeding practices. In 2013, due to widespread TB, other sanitary problems and selling of deer to properties in different Brazilian states, the official veterinary service, through a court order, demanded the closure of the park to visitations and halting of animal trade. From 2013 onwards, many tuberculin skin tests were performed in deer, cattle and buffalo. However, as the PNCEBT solely covers the latter two species, only these were mandatorily culled after testing positive. The deer tested positive by TST in 2013, 2014 and 2015, and recommendations to contain the outbreak were made to the park. In 2018, after the official veterinary service noted the park did not carry on with the recommendations, the owners of the park proceeded to depopulate the deer following a judicial court order. This judicial court order used as basis the unlawful interstate trade of potentially infected animals. Tissue from necropsy of the two llamas and the culled deer were the only animal samples ever subjected to *M. bovis* culture. A more complete history of all TB cases of the park is described in Supporting Information.

3.2 | Phylogenetic reconstruction of *M. bovis* from the safari park

Out of the 19 *M. bovis* isolates obtained from deer, two sequenced genomes were excluded because they presented only 35% and 43% coverage against *M. tuberculosis* H37Rv. All remaining genomes were confirmed to be *M. bovis* based on the absence of RD4 (i.e., region is deleted) and presence of RD1 (i.e., region is not deleted). Interestingly, one genome (TB002S2L) presented 35.77% of heterogeneous SNPs, suggesting the existence of a mixed strain infection in a deer; this genome was excluded from further analysis. The remaining 16 *M. bovis* genomes from deer and both genomes obtained from the llamas were further used to construct a ML phylogenetic tree and a PCA using a core-SNP matrix (Figure 3).

The phylogenetic reconstruction suggests the existence of four distinct clusters circulating in the safari park. The most basal of these clusters is formed by a single *M. bovis* isolate from a deer (TB013S13L, cluster 4, purple, Figure 3), while the three other clusters are formed by seven *M. bovis* from deer (cluster 1, green, Figure 3), four *M. bovis* isolates from llamas and deer (cluster 2, black, Figure 3) and six *M. bovis* isolates from deer (cluster 3, blue, Figure 3). The PCA analysis indicates the separation of at least three clusters, while two of them appeared very closely related (green and black), as also depicted in the phylogenetic tree (Figure 3, Figure S1). Pairwise SNP comparison of the clusters indicate lower SNP distances in cluster 1, followed by cluster 3 and 2 (Figure S2). All *M. bovis* isolates of the safari park carry the European 2 marker, being thus identified as lineage Lb3 according to recent classification (Zimpel et al., 2020). Interestingly, the *M. bovis* spoligotype patterns were the same, SB0295, for the isolates of all animals of the safari park, except for the single *M. bovis* isolate of a deer from cluster 4 (SB0121) (Figure 3).

3.3 | *M. bovis* from the safari park and cattle farms

We then evaluated the 18 *M. bovis* genomes of deer and llamas in the context of 71 additional genomes obtained from cattle herds located from 37 to 308 km away of the safari park. The phylogenetic tree and associated CCs, lineages and spoligotype patterns are shown in Figure 4. While 17 genomes of *M. bovis* from the safari park were part of the same clade (which includes clusters 1, 2 and 3 of the safari park), the most distantly related genome TB013S13L branched into a separated clade with other *M. bovis* isolates from cattle. In contrast, three *M. bovis* genomes from cattle herds appeared within the same main

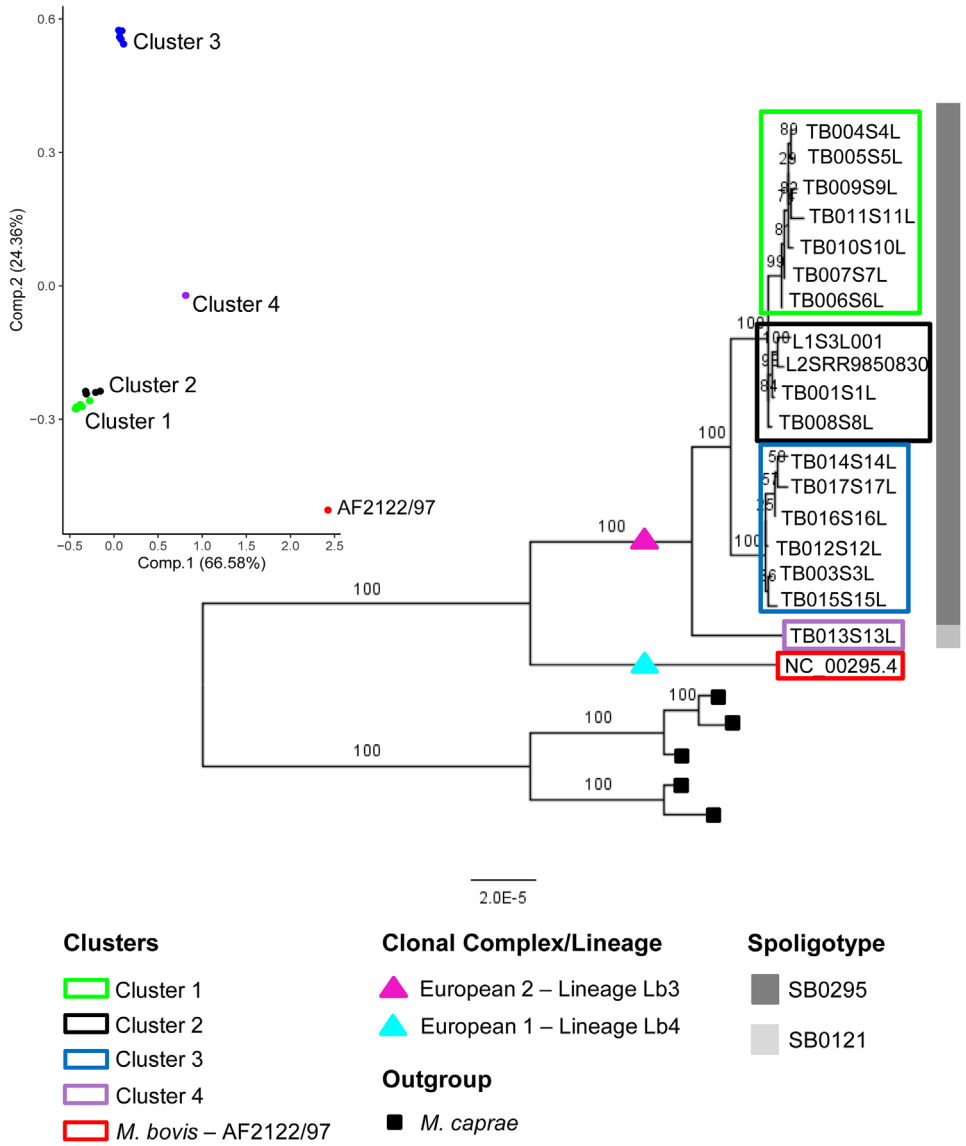

FIGURE 3 Phylogenetic reconstruction and principal component analysis (PCA) of 18 *Mycobacterium bovis* genomes obtained from the safari park. *Mycobacterium bovis* genomes starting with "TB0" were obtained from deer ($n = 16$) and genomes starting with 'L' were obtained from llamas ($n = 2$). Phylogenetic clusters are shown in the PCA using the same colours. NC_00295.4 is the reference genome of *M. bovis* AF2122/97. Phylogenetic tree was constructed from a core-SNP matrix using maximum likelihood in IQ-Tree with 1,000 bootstrap replicas. *Mycobacterium caprae* strains were used as outgroup. Lineages were identified according to Zimpel et al. (2020). Horizontal bar shows substitutions per nucleotide

clade of the deer and llamas (clusters 1 and 2) from the safari park. Although genomes carrying the same spoligotype pattern tended to cluster, different clusters of the same spoligotype pattern (e.g., SB0295 and SB0121) appeared dispersed throughout the phylogenetic tree (Figure 4). Both lineages Lb3 and Lb4, represented by CC markers of Eu2 and Eu1, respectively, were detected in the dataset, with Lb4 constituted by *M. bovis* isolates from cattle farms only.

3.4 | Dating estimates

The park was inaugurated in 1977 and personal communications from a previous employee indicates that TB cases in the park date back from the 1980s. Thus, to better elucidate *M. bovis* introduction, we estimated the ages of the most recent common ancestor (MRCAs) of the *M. bovis* strains circulating in the safari park (Table 1). Accordingly, the dating estimates of the MRCAs of each of the three *M. bovis* clusters (1 to 3, Figures 3 and 4) from the safari park ranged from 10 to 40 years before present (BP) (Table 1). In addition, the MRCA of clusters 1 and 2 dated from 23 to 47 years ago, which is also within the time frame of the park existence. In contrast, the MRCA of clusters 1, 2 and 3 dated from 50 to 103 years ago, which is older than the park inauguration. Thus, for clusters 1 and 2, and for cluster 3, it is possible that they are products of two different introduction events at the beginning of the safari park, evolving into different strains over time through persistent animal to animal transmission. Finally, another introduction event

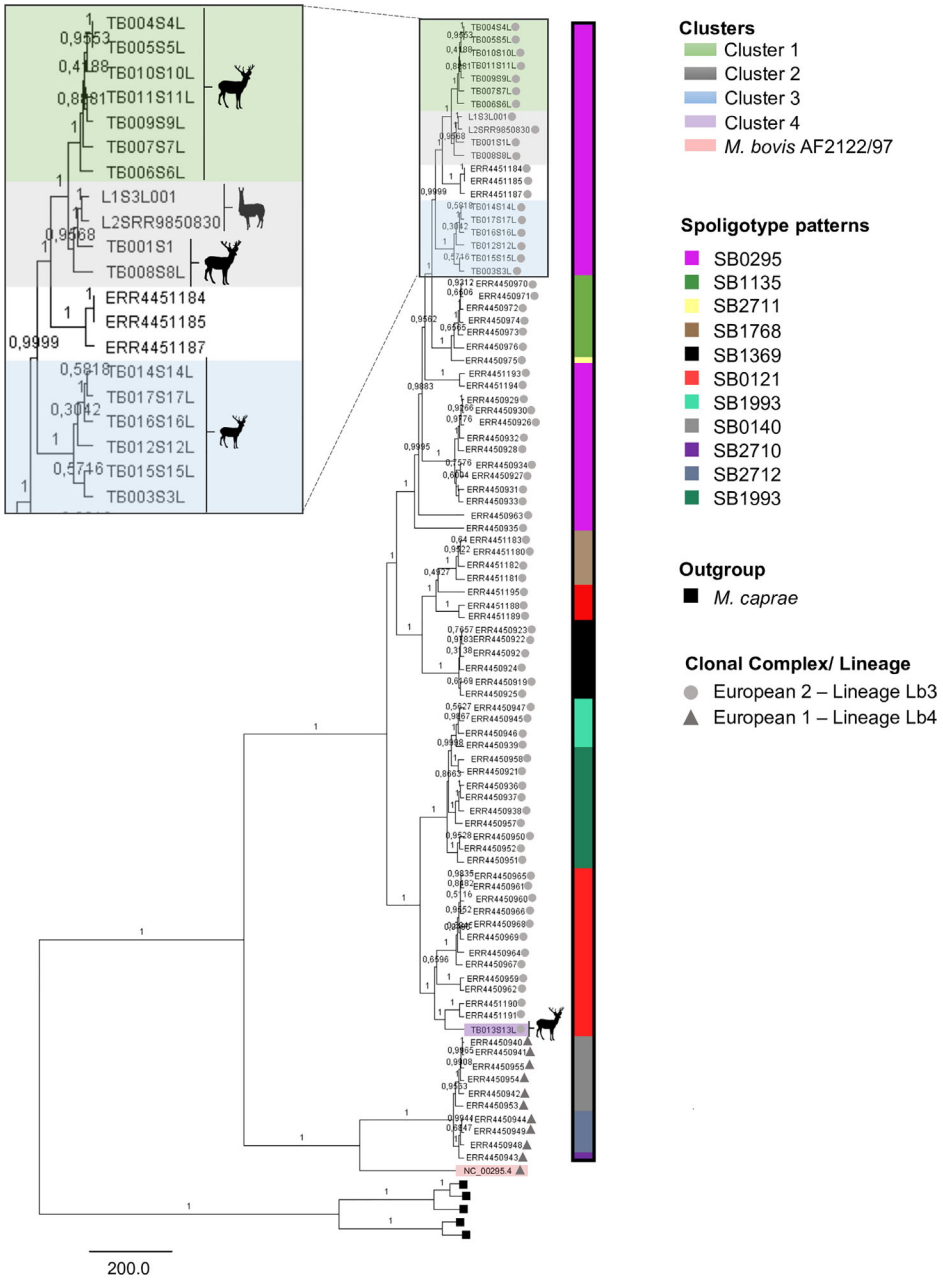

FIGURE 4 Bayesian phylogenetic tree of *Mycobacterium bovis* genomes from the safari park ($n = 18$) and cattle herds ($n = 71$) of Rio Grande do Sul. Phylogenetic tree was generated in BEAST v1.10.4, using a core SNP-matrix. Horizontal bar indicates number of SNPs (single nucleotide polymorphisms). *Mycobacterium caprae* strains are used as outgroup.

pertains to isolate TB013S13L, with a MRCA dating back from 25 to 77 years ago. The MRCA of *M. bovis* and *M. caprae* was dated from 630 to 1,644 years BP, similar to a previous study (Zimpel et al., 2020).

3.5 | Transmission links and minimum spanning tree

Next, we used the core-SNP matrix of all *M. bovis* isolates to infer a distance matrix (Figures S3 and S4, Table S2) and a minimum spanning tree of the dataset (Figure 5). Traditionally, SNP distances between genomes have been used to infer transmission of *M. tuberculosis* between individuals. Thresholds varying from 5 to 12 SNPs have been proposed to determine if two genomes are from the same *M. tuberculosis* strain (Meehan et al., 2019). By using a more stringent threshold of ≤ 5 SNPs to detect recent transmission events, two transmission links were detected in the animals from the safari park (Table S3). Seven additional 'suspected' transmission links using a more conservative threshold of 6 to 12 SNPs were also detected. Together, these nine putative transmission links involve five deer and constitute a transmission cluster as detected in the MST analysis (Figure 5). The SNP distances between these genomes were: 4 ($n = 2$), 8 ($n = 1$), 10 ($n = 3$)

TABLE 1 Dating estimates of *Mycobacterium bovis* clusters from the safari park

M. bovis clusters	Dating estimates (years before present)	Approximate year
MRCA of cluster 1	10–26	1992–2008
MRCA of cluster 2	18–39	1979–2000
MRCA of cluster 3	15–40	1978–2003
MRCA of clusters 1 and 2	23–47	1971–1995
MRCA of clusters 1, 2 and 3	50–103	1915–1968
MRCA of TB013S13L (cluster 4)	25–77	1993–1941
MRCA of *M. bovis* from llamas	10–21	1997–2008

Abbreviation: MRCA, most recent common ancestor.

and 12 ($n = 3$) (Table S3). No transmission links between llamas and deer and between the safari park and cattle herds were detected using this approach. The two closest *M. bovis* genomes of cattle to *M. bovis* genomes of the safari park differed by 92 and 64 SNPs, which are five to seven times higher than the SNP cut-off to infer recent transmission (Figure 5). Along with the dating estimates, these results indicate that multiple *M. bovis* strains were circulating in the safari park at the time of sample collection, resulting from at least three different past introductions.

Interestingly, multiple transmission links ($n = 48$; being 2 with ≤ 5 SNPs) were detected involving cattle from vicinity herds, represented by five transmission clusters in the MST analysis (Figure 5, Table S3). The cattle transmission involved seven farms, the vast majority (40/48; 83.3%) of the transmission pairs occurred within the same farm, and all but two pairs of genomes shared the same spoligotype pattern (Table S3). The eight between-farm transmission events are further supported by field epidemiological data indicating animal movements between these farms (data not shown) (Rodrigues et al., 2021).

4 | DISCUSSION

In this study, we describe an outbreak of tuberculosis caused by *M. bovis* in a Brazilian safari park that lasted for at least 15 years. This outbreak was a result of multiple pathogen introductions and

FIGURE 5 Minimum spanning tree of 18 *Mycobacterium bovis* genomes from the safari park and 71 *M. bovis* from cattle herds of Rio Grande do Sul state. Nodes are *M. bovis* genomes and edges represent the number of SNPs between two genomes (number of SNPs indicated on each edge). Nodes in orange represent possible transmission links using a SNP (single nucleotide polymorphism) cutoff of 12. Genomes of *M. bovis* starting with TB0 (deer) and L (llama) are from the safari park, while genomes of *M. bovis* starting with ERR are from cattle herds.

culminated with the death of many animal specimens. There are two main reasons that can explain why this outbreak was so severe. First, starting in 2005 the park passed through management changes and financial hurdles that led to animal overcrowding and malnutrition. Many animals of different species shared a small enclosure and feeding practices were not adequate to serve them all. Overcrowding and malnutrition are known risk factors of bTB (Pollock & Neill, 2002). Second, the park owners often acquired and introduced animals of unknown bTB status (i.e., without diagnostic testing) into the premises, particularly cattle and buffalo. Collectively, these factors may have facilitated pathogen introduction and spread in the property as well as the development of clinical disease by the wild animals.

Our results suggest two possible timeline scenarios for the dynamics of this bTB outbreak. One scenario involves at least three different *M. bovis* introductions occurring in the time period between the park inauguration in 1977 up until around 2003. The founding *M. bovis* subsequently evolved into many different sequence types over time, fuelled by persistent animal to animal transmission. These sequence types were likely circulating at low levels or asymptomatically until the above-described conditions of overcrowding and malnutrition became conducive of a deadly outbreak. As *M. bovis* is endemic in the region (5.2% of infected herd prevalence) (Queiroz et al., 2016), a second scenario involves the introduction of many different sequence types over the years, possibly through cattle, contributing to the high *M. bovis* diversity observed in the property. Although cattle and wildlife were separated by a fence, escape and mixing of animals in both enclosures were reportedly common events. Alternatively, pathogen introduction may have occurred with the acquisition of specimens of infected wild animals, since TB cases occur in wild animals in captivity in Brazil (Ikuta et al., 2018; Murakami et al., 2012; Rocha et al., 2011; Zimpel et al., 2017).

As a wild animal reservoir of bTB has not been identified in Brazil so far, in its inception the PNCEBT was designed to cover cattle and buffalo only. Hence notifications of TB cases in wildlife are not mandatory, diagnostics are not standardized, and there are no official guidelines for outbreak resolution or prevention in wildlife. These factors likely influenced the course of the outbreak described herein. Thus, we believe the inclusion of wildlife in the PNCEBT can bring great benefits in terms of animal welfare, while increasing biosafety in zoos and parks and allowing active surveillance of wild animals. In addition, regulation of the diagnosis of TB in wild animals would have an impact on reducing outbreaks that may occur through sale or trade of infected animals. The many outbreaks seen in Brazilian captive populations over the years (Ikuta et al., 2018; Murakami, Monego, Ho, Gibson, De Castro Vilani et al., 2012; Murakami, Monego, Ho, Gibson, Javorouski et al., 2012; Rocha et al., 2011; Zimpel et al., 2017), along with the one reported herein, should form the rationale necessary to develop this nationwide plan to control and report TB in wildlife, as also suggested previously (Valvassoura & Ferreira Neto, 2014). Notwithstanding, for these policies to become effective, it is necessary to integrate federal and state surveillance services and environmental regulatory agencies.

Our study adds to the importance of evaluating potential paths of transmission to understand bTB outbreaks. Using phylogenetic analysis and *M. tuberculosis*-based cut-offs for the number of SNPs, many different sequence types of *M. bovis* were found circulating in the park, with nine (two confirmed and seven suspected) recent transmission events involving five deer. The low SNP distance (4, 8, 10 or 12 SNPs) between these five genomes suggests that the same *M. bovis* strain was transmitted from one animal to another, and the amount of accumulated genetic changes is just a reflection of microevolution. In addition, one of the animals was co-infected with multiple strains, as it presented many heterogeneous SNPs. This mixed infection occurs when the individual is exposed to a single infection event carrying multiple strains, or to several infection events with different strains throughout its life, resulting in a superinfection. Such condition is normally seen in conditions of high disease endemicity, as described in this park.

Using the SNP-cutoff approach, recent transmission events between the surveyed cattle farms and the safari park were not observed in this study. It is possible that the sampling of cattle farms was not comprehensive enough to capture the true *M. bovis* diversity of Rio Grande do Sul state. The origin of the cattle maintained at the safari park was unknown to the authors of this study; thus, it is possible that the region from which they originated was not covered in the cattle sampling, which may include out of the state farms. Alternatively, the *M. bovis* strains infecting deer and llamas have been introduced a long time ago in the park (as suggested by the dating analysis) and evolved in geographic isolation. Unfortunately, without more comprehensive and retrospective data, it is not possible to pinpoint the exact origin of the *M. bovis* strains circulating in this park.

Although commonly used as a measure of *M. tuberculosis* or *M. bovis* transmission between individuals, the SNP cutoff approach was never standardized for *M. bovis*, is sensitive to the pipeline used to detect SNPs (Guimaraes & Zimpel, 2020), and also a simplistic approach to follow the complex evolution of tuberculous mycobacteria (Menardo et al., 2019). The BEAST analysis performed herein rejected a strict clock evolution model of *M. bovis*, corroborating previous studies on *M. bovis* (Crispell et al., 2019; Salvador et al., 2019; Zimpel et al., 2020) and *M. tuberculosis* (Menardo et al., 2019; Rutaihwa et al., 2019). We are also unaware if evolutionary rates of *M. bovis* are influenced by the disease form (e.g., latent *versus* clinical disease) or host species. All these factors must be taken into consideration when interpreting results from this study. However, to make transmission inferences more robust, other genotyping markers and epidemiological data can be simultaneously considered (Kao et al., 2016). We showed that 96.5% of the possible transmission pairs presented the same spoligotype pattern and most of the cattle links occurred between animals of the same farm (Table S3), providing further support to the genomic approach taken herein to infer transmission events, even when using a more conservative SNP-threshold. Nevertheless, we believe research should be continued to optimize the methodology to infer transmission of *M. bovis* strains and to analyze how these can be compared to traditional genotyping techniques such as spoligotyping and MIRU-VNTR.

This study has limitations. Unfortunately, *M. bovis* isolates from all other animals that died during the outbreak were not obtained, precluding our ability to evaluate the true genetic diversity of the pathogen in the park. We were also unable to control sample

collection at the slaughterhouse where deer were culled, thus preventing a better description of the lesions found, number of affected animals and host species. And finally, we did not have access to records of animal introductions that occurred in the park, impeding a more comprehensive analysis of pathogen introduction.

5 | FINAL CONSIDERATIONS

By combining epidemiological history and WGS of *M. bovis* isolates, we show that the increase in fatal cases observed in the safari park coincided with changes in populational density and nutritional status, and that many pathogen introductions occurred over time. These observations demonstrate that wildlife populations found to be chronically infected and frequently asymptomatic may suddenly suffer from acute, fatal disease following populational disturbances, such as the ones observed herein. The negligence of successive pathogen introductions likely potentiated the outbreak, contributing to disease spread in different animal species. The *M. bovis* strains circulating in the park were classified as Lb3 (CC Eu2), a common lineage found in Brazil, and share phylogenetic relationships with extant *M. bovis* strains circulating in farmed cattle. It is possible that at some point in time, transmission occurred between cattle and the wildlife maintained in the safari park. However, current data cannot identify the exact origin of the *M. bovis* strains circulating in the park. The use of WGS to delineate the history of this outbreak highlights the importance of genomic surveillance to follow disease introduction into animal populations and transmission between individuals.

With this study we make a pledge for action. It is imperative that reporting of TB cases in wildlife, captive or free ranging, becomes mandatory in Brazil as to understand the true size of the problem. This should follow increased investments in pathogen identification, as wildlife are affected by both *M. bovis* and *M. tuberculosis* (Murakami, MonegoHo, Gibson, Javorouski et al., 2012). Finally, a national plan for control of TB in wildlife should be designed to incorporate specific guidelines to contain and solve outbreaks and prevent possible zoonotic transmission to veterinarians and caretakers, considering species-specific conditions and difficulties in diagnosing the disease in wild animals, particularly when dealing with endangered species.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

Animal tissue samples were obtained at necropsy (llamas) or at slaughterhouse (deer). No animal was subjected to experimentation or was euthanized as per requirement of this study.

DATA AVAILABILITY STATEMENT

Data is available from SRA (Sequence Read Archive), NCBI under accession numbers: SRR13015794 through SRR13015810, SRR7693912, SRR7693877.

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