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XXXVIII CYCLE

Biomedical, Immunologicals and Epidemiological Studies on *Leishmania*

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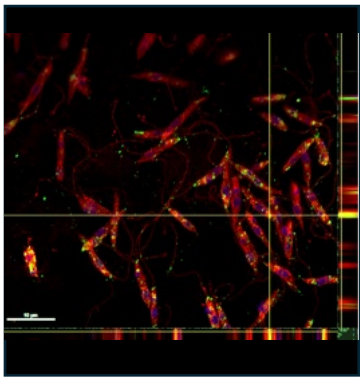
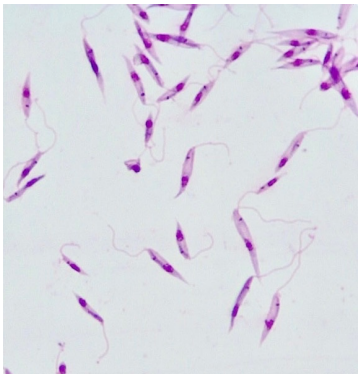
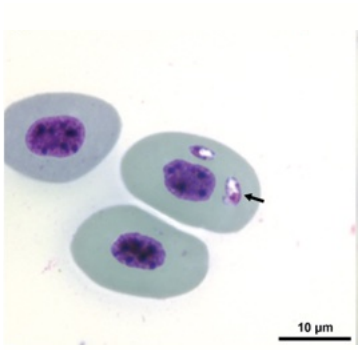

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UNIVERSITÀ DEGLI STUDI DI MILANO

UNIVERSITÀ DEGLI STUDI DI MILANO

CORSO DI DOTTORATO in ENVIRONMENTAL SCIENCES XXXVIII cycle

DIPARTIMENTO DI SCIENZE AMBIENTALI

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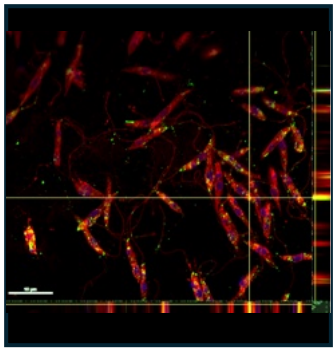
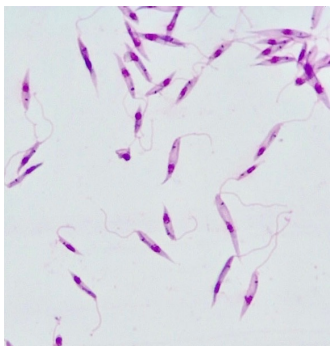
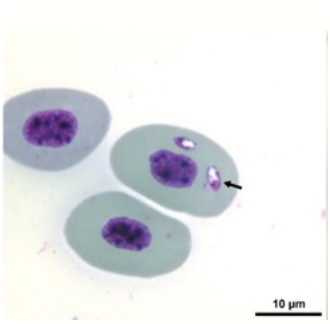

A.A. 2025/2026

1 Abstract of the thesis

Leishmania tarentolae is a non-pathogenic protozoan parasite generally associated with reptiles, which has recently emerged as a versatile model in biothecnological and biomedical research. It is characterized by rapid growth rate, low maintenance cost, and can be handled under Biosafety Level 1 (BSL-1) conditions, making it a safe and accessible laboratory system. Despite being non-pathogenic, *L. tarentolae* retains molecular and biological features common to pathogenic *Leishmania* species, representing a relevant model for host–parasite interaction studies.

L. tarentolae is increasingly used as a eukaryotic expression platform for the production of heterologous protein. Unlike *Escherichia coli*, it performs mammalian-like post-translational modifications, including glycosylation, while avoiding the hyper-mannosylation introduced by yeast systems, which may compromise protein folding and functionality for biomedical use. Compared with mammalian cell cultures, *L. tarentolae* offers lower operational costs, high scalability, and reduced biosafety constraints. These combined features reinforce its attractiveness as a cell factory for biotechnological and therapeutic applications.

This PhD research investigates the technological, immunological and diagnostic potential of *L. tarentolae* through four major lines of investigation:

- Development of a chemically defined medium for *L. tarentolae* growth, enabling its conformity with biomedical research guidelines;
- Application of *L. tarentolae* as a mucosal delivery system for recombinant antigens, demonstrated with the experimental SARS-CoV-2 vaccine LeCoVax-2;
- Exploitation of *L. tarentolae* as a mucosal vaccine vector, to explore its potential to elicit a specific immune response.
- Evaluation of host–parasite interactions, focusing on primary canine macrophages cell line.

In addition, considering the current limited knowledge on the distribution of *Leishmania tarentolae*, this thesis also investigates the epidemiology and diagnostic detection of this parasite in northern Italy. Particular attention is given to the possible involvement of sand fly *Sergentomyia minuta*, which is traditionally considered the vector of *L. tarentolae* and has previously tested positive for *L. infantum* DNA. Furthermore, *L. tarentolae* DNA detection in human and canine blood raises questions about the potential expansion of its host range.

Considering this, the thesis also examined the presence of *Sauroleishmania* in additional hosts, including fire salamanders and geckos, while focusing on the role of synanthropic reptiles. This investigation is particularly relevant considering that the close association of these hosts with human environments and domestic animals may facilitate the circulation of *Leishmania* parasites.

Basing on this evidences, the research conducted in this thesis focuses also on:

- The development of a highly sensitive method for simultaneous detection of the non-pathogenic *L. tarentolae* and the pathogenic *L. infantum* in sand flies and reptile blood samples.
- A screening of *L. tarentolae* and *L. infantum* in synanthropic reptiles and in sand fly vectors in the northern Italy.
- Investigation of the host range of *Sauroleishmania* species in non-traditional hosts, including fire salamanders and African geckos.

The results obtained in these studies are presented in eight publications included in the thesis:

Article 1: A novel chemically defined medium for the biotechnological and biomedical exploitation of the cell factory *Leishmania tarentolae*.
[published in: Scientific Repors].
This study describes the development of a medium without animal supplementation for the cultivation of *L. tarentolae* cultures, enabling its applications in the biomedical field. The chemically-defined medium was also tested and validated on recombinant *L. tarentolae* clones, higliting its potential for the production of recombinant proteins and its exploitation as cell factory.

Article 2: Efficacy of mucosal vaccination using a protozoan parasite as a vehicle for antigen delivery: IgG and neutralizing response after rectal administration of LeCoVax-2, a candidate vaccine against COVID-19.
[published in: Pharmacological Research]

This study investigates the potential of recombinant *L. tarentolae* clones as vehicle for mucosal vaccine delivery, exploiting an engineered *L. tarentolae* clone expressing a SARS-CoV-2 antigen. This article aims to explore the possibility of inducing systemic and mucosal immune reponses, including IgG and neutralizing anitbodies, through a rectal administration. This innovative strategy contributes to revealing the potential versatility of *L. tarentolae* platform.

Article 3: Rectal Administration of *Leishmania* Cells Elicits a Specific, Th1-Associated IgG2a Response in Mice: New Perspectives for Mucosal Vaccination against leishmaniasis**, after the Repurposing of a Study on an Anti-Viral Vaccine Candidate.**

[published in: Tropical Meidcin and Infectious Disease]

This study investigates *L. tarentolae* as a mucosal vaccine vector against leishmaniasis via rectal administration in a murine model, demonstrating its ability to induce a robust Th1-oriented immune response, which is associated with protective immunity against *Leishmania*. These findings open new avenues for alternative vaccination routes.

Article 4: Intracellular persistence of *Leishmania tarentolae* in primary canine macrophage cells.

[published in: Acta Tropica]

This study focused on the interaction between primary canine macrophages and L. *tarentolae*, exploring its intracellular behaviour, evaluating the possible cytopathic effects and the host immune response. The results indicate that *L. tarentolae* is non-pathogenicity and can persist intracellularly without inducing cytopathic effects, higliting its potential as a versatile platform for biomedical applications.

Article 5: Development of a novel ddPCR assay for the simultaneous detection of the protozoan parasites *Leishmania* infantum and *Leishmania tarentolae*. [published in: Parasites&Vectors]

This study describes the development of a digital droplet PCR (ddPCR) assay for the simoultaneous detection of the non-pathogenic *L. tarentolae* and the pathogenic *L. infantum*. This research aims to develope an highly sensitive and specific assay that can provide and higher accuracy in *Leishmania* diagnostics avoiding cross-reaction results.

Article 6: Sand Fly Fauna and Prevalence of *Leishmania* spp. in a Newly Investigated Area of Northern Italy: Emerging Epidemiological Scenarios? [published in: Transboundaries and Emerging Diseases]

This study investigates the circulation and distribution of *L. tarentolae* and *L. infantum* in Northern, focusing of sand flies and reptile blood samples. A ddPCR developed during the PhD period was employed to obtain a higly sensitive detection. This research evaluate also the potential role of synanthropic reptiles as reservoirs, and assess emerging epidemiological scenarios for *Leishmania* transmission.

Articole 7: Beyond reptiles: the fire salamander as a potential host for *Leishmania* (*Sauroleishmania*) *tarentolae* [published in International Journal of Parasitology: Parasites and Wildlife]

This study investigates the potential role of fire salamanders as hosts for *Leishmania (Sauroleishmania) tarentolae*, expanding the understanding of the parasite's host range. The research evaluates the presence of the parasite in non-traditional hosts, considering the ecological implications for parasite circulation and transmission dynamics.

Article 8: Unveiling *Sauroleishmania* in southern Africa: An investigation and supplementary description of *Leishmania* (*Sauroleishmania*) zuckermani from the gecko host *Chondrodactylus bibronii* [published in International Journal for Parasitology: Parasites and Wildlife]

This study presents a work carried out during a three-months research period abroad at the University of Free State (Bloemfontein, South Africa), exploring the distribution and host associations of *Sauroleishmania* in African geckos. The research provides insights into the ecology and host specificity of *Sauroleishmania* species, highlighting potential new reptile hosts and contributing to the understanding of parasite circulation in non-European regions.

In conclusion, the results presented in this thesis provide a comprehensive overview of *L. tarentolae* as both an emerging biotechnological tool and a parasite of epidemiological relevance. The study highlights the potential of *L. tarentolae* as a safe and scalable platform for recombinant protein production and mucosal vaccine delivery, while also exploring its immunomodulatory properties and host–parasite interactions in mammalian cells.

Furthermore, this thesis addressed the development of sensitive molecular tools for the simultaneous detection of *L. tarentolae* and *L. infantum*. Investigations on sand flies and synanthropic reptiles further contribute to understanding parasite circulation and potential transmission pathways in Northern Italy. Finally, studies on non-traditional hosts, including fire salamanders and African geckos, expand our knowledge of *Sauroleishmania* host range and distribution.

Collectively, these studies underscore the dual significance of *L. tarentolae* as both a biotechnological resource and a topic of epidemiological interest.

Riassunto della tesi

Leishmania tarentolae è un protozoo non patogeno generalmente associato ai rettili, recentemente emerso come modello versatile nella ricerca biotecnologica e biomedica. È caratterizzato da un rapido tasso di crescita, bassi costi di mantenimento e può essere manipolato in condizioni di Biosicurezza di Livello 1 (BSL-1), rendendolo un sistema laboratoristico sicuro e accessibile. Nonostante la non patogenicità, *L. tarentolae* conserva caratteristiche molecolari e biologiche comuni alle specie patogene di *Leishmania*, rappresentando un modello rilevante per lo studio delle interazioni ospite-parassita.

L. tarentolae è sempre più utilizzata come piattaforma eucariotica per l'espressione di proteine eterologhe. A differenza di *Escherichia coli*, effettua modificazioni post-traduzionali di tipo mammifero, inclusa la glicosilazione, evitando l'iper-mannosilazione introdotta dai sistemi di lievito, che può compromettere il folding proteico e la funzionalità biomedica. Rispetto alle colture cellulari di mammifero, *L. tarentolae* offre costi operativi ridotti, alta scalabilità e minori vincoli di biosicurezza. Queste caratteristiche combinate ne rafforzano l'attrattiva come "cell factory" per applicazioni biotecnologiche e terapeutiche.

Questa ricerca di dottorato indaga il potenziale tecnologico, immunologico e diagnostico di *L. tarentolae* attraverso quattro linee principali di indagine:

- Sviluppo di un terreno chimicamente definito per la crescita di *L. tarentolae*, consentendo la conformità alle linee guida per la ricerca biomedica;
- Applicazione di *L. tarentolae* come sistema di somministrazione mucosale di antigeni ricombinanti, dimostrato mediante il vaccino sperimentale SARS-CoV-2 LeCoVax-2;
- Sfruttamento di *L. tarentolae* come vettore vaccinale mucosale, per esplorarne il potenziale nell'indurre una risposta immunitaria specifica;
- Valutazione delle interazioni ospite–parassita, con particolare focus sulla linea cellulare di macrofagi primari canini.

Inoltre, considerata la conoscenza ancora limitata sulla distribuzione di *Leishmania tarentolae*, questa tesi indaga anche l'epidemiologia e la rilevazione diagnostica del parassita nel Nord Italia. Particolare attenzione è rivolta al possibile coinvolgimento del flebotomo *Sergentomyia minuta*, tradizionalmente considerato vettore di *L. tarentolae* e risultata precedentemente positiva per DNA di *L. infantum*. Inoltre, la presenza di DNA di *L. tarentolae* rilevata in campioni di sangue umano e canino solleva interrogativi sull'eventuale ampliamento del suo range di ospiti.

In questo contesto, la tesi ha esaminato anche la presenza di *Sauroleishmania* in ospiti aggiuntivi, tra cui salamandra pezzata e gechi, con particolare attenzione al ruolo dei rettili sinantropici. Tale indagine è particolarmente rilevante considerando che la stretta associazione di questi ospiti con ambienti antropizzati e animali domestici può facilitare la circolazione dei parassiti *Leishmania*.

Sulla base di queste evidenze, la ricerca condotta in questa tesi si concentra anche su:

- Lo sviluppo di un metodo altamente sensibile per la rilevazione simultanea della non patogenica *L. tarentolae* e della patogena *L. infantum* in campioni di flebotomi e sangue di rettili;
- Uno screening di *L. tarentolae* e *L. infantum* in rettili sinantropici e flebotomi nel Nord Italia;
- L'indagine sul range di ospiti delle specie *Sauroleishmania* in ospiti non tradizionali, tra cui salamandra pezzata e gechi africani.

I risultati ottenuti in questi studi sono presentati in otto pubblicazioni incluse nella tesi:

Articolo 1: A novel chemically defined medium for the biotechnological and biomedical exploitation of the cell factory *Leishmania tarentolae*.

[pubblicato in: Scientific Reports]

Questo studio descrive lo sviluppo di un terreno privo di supplementi animali per la coltivazione di *L. tarentolae*, consentendo le applicazioni nel campo biomedico. Il terreno chimicamente definito è stato testato e validato su cloni ricombinanti di *L. tarentolae*, evidenziandone il potenziale per la produzione di proteine ricombinanti e il suo impiego come cell factory.

Articolo 2: Efficacy of mucosal vaccination using a protozoan parasite as a vehicle for antigen delivery: IgG and neutralizing response after rectal administration of LeCoVax-2, a candidate vaccine against COVID-1G.

[pubblicato in: Pharmacological Research]

Questo studio esplora il potenziale dei cloni ricombinanti di *L. tarentolae* come veicolo per la somministrazione mucosale di vaccini, utilizzando un clone ingegnerizzato che esprime un antigene SARS-CoV-2. L'articolo valuta la possibilità di indurre risposte immunitarie sistemiche e mucosali, incluse IgG e anticorpi neutralizzanti, mediante somministrazione rettale. Questa strategia innovativa evidenzia la versatilità della piattaforma *L. tarentolae*.

Articolo 3: Rectal Administration of *Leishmania* Cells Elicits a Specific, Th1-Associated IgG2a Response in Mice: New Perspectives for Mucosal Vaccination against leishmaniasis, after the Repurposing of a Study on an Anti-Viral Vaccine Candidate.
[pubblicato in: Tropical Medicine and Infectious Disease]
Questo studio analizza *L. tarentolae* come vettore vaccinale mucosale contro la leishmaniosi in modello murino tramite somministrazione rettale, dimostrando la capacità di indurre una robusta risposta immunitaria orientata Th1, associata a immunità protettiva contro *Leishmania*. I risultati aprono nuove prospettive per vie alternative di vaccinazione.

Articolo 4: Intracellular persistence of *Leishmania tarentolae* in primary canine macrophage cells.
[pubblicato in: Acta Tropica]
Questo studio si concentra sull'interazione tra macrofagi primari canini e *L. tarentolae*, esplorandone il comportamento intracellulare, valutando potenziali effetti citopatici e la risposta immunitaria dell'ospite. I risultati indicano che *L. tarentolae* è non patogena e può persistere intracellularmente senza effetti citopatici, evidenziandone il potenziale come piattaforma versatile per applicazioni biomediche.

Articolo 5: Development of a novel ddPCR assay for the simultaneous detection of the protozoan parasites *Leishmania* infantum and *Leishmania tarentolae*.
[pubblicato in: Parasites & Vectors]
Questo studio descrive lo sviluppo di un saggio di digital droplet PCR (ddPCR) per la rilevazione simultanea della non patogenica *L. tarentolae* e della patogena *L. infantum*, con l'obiettivo di ottenere un test altamente sensibile e specifico, riducendo falsi positivi dovuti a cross-reattività.

Articolo 6: Sand Fly Fauna and Prevalence of *Leishmania* spp. in a Newly Investigated Area of Northern Italy: Emerging Epidemiological Scenarios?
[pubblicato in: Transboundary and Emerging Diseases]
Questo studio indaga la circolazione e distribuzione di *L. tarentolae* e *L. infantum* nel Nord Italia, con focus su flebotomi e campioni di sangue di rettili. È stata impiegata la ddPCR

sviluppata durante il periodo di dottorato per una rilevazione altamente sensibile. La ricerca valuta il ruolo potenziale dei rettili sinantropici come serbatoi e propone scenari epidemiologici emergenti per la trasmissione di *Leishmania*.

Articolo 7: Beyond reptiles: the fire salamander as a potential host for *Leishmania* (*Sauroleishmania*) *tarentolae*

[pubblicato in: International Journal of Parasitology: Parasites and Wildlife]

Questo studio esplora il ruolo potenziale della salamandra pezzata come ospite per *L. (Sauroleishmania) tarentolae*, ampliando la conoscenza riguardo gli range di ospiti del parassita. La ricerca valuta la presenza del parassita in ospiti non tradizionali, considerando le implicazioni ecologiche per la circolazione e la trasmissione del parassita.

Articolo 8: Unveiling *Sauroleishmania* in southern Africa: An investigation and supplementary description of *Leishmania* (*Sauroleishmania*) zuckermani from the gecko host *Chondrodactylus bibronii*

[pubblicato in: International Journal for Parasitology: Parasites and Wildlife]

Questo studio presenta un lavoro condotto durante un periodo di ricerca di tre mesi presso la University of Free State (Bloemfontein, Sudafrica), esplorando distribuzione e associazioni ospite di *Sauroleishmania* in gechi africani. La ricerca fornisce approfondimenti sull'ecologia e la specificità degli ospiti delle specie di *Sauroleishmania*, evidenziando nuovi potenziali ospiti rettili e contribuendo alla comprensione della circolazione del parassita in aree non europee.

In conclusione, i risultati presentati in questa tesi offrono una panoramica completa su *L. tarentolae* come strumento biotecnologico emergente e parassita di rilevanza epidemiologica. Lo studio evidenzia il potenziale della piattaforma *L. tarentolae* come sistema sicuro e scalabile per la produzione di proteine ricombinanti e la somministrazione mucosale di vaccini, esplorandone anche le proprietà immunomodulatorie e le interazioni ospite-parassita in cellule di mammifero.

Inoltre, la tesi ha affrontato lo sviluppo di strumenti molecolari sensibili per la rilevazione simultanea di *L. tarentolae* e *L. infantum*. Le indagini su flebotomi e rettili sinantropici contribuiscono ulteriormente alla comprensione della circolazione del parassita e dei potenziali percorsi di trasmissione nel Nord Italia. Infine, gli studi su ospiti non tradizionali,

comprese salamandre di fuoco e gechi africani, ampliano le conoscenze sul range e sulla distribuzione degli ospiti di *Sauroleishmania*.

Collettivamente, questi studi evidenziano la duplice rilevanza di *L. tarentolae* come risorsa biotecnologica e come oggetto di interesse epidemiologico.

2 Leishmaniasis

2.1 Epidemiology

Leishmaniases, also known as leishmaniosis, are vector-borne parasitic diseases caused by protozoa of the genus *Leishmania* within the order Kinetoplastida. The infection is transmitted through the bite of infected female phlebotomine sand flies, taxonomically classified within Diptera, Psychodidae, and Phlebotominae. To date, more than fifty species of *Leishmania* have been identified, with approximately twenty recognized as agents of human disease (Akhoundi et al. 2016). As a result of its persistent burden, limited visibility, and insufficient investment in control strategies, the disease is identified by the World Health Organization as a Neglected Tropical Disease. Current global estimations indicate that several hundred thousand new cases occur annually, reflecting substantial underreporting (WHO 2022; 'CDC - DPDx - leishmaniasis' 2019).

Clinically, leishmaniasis includes a spectrum of clinical manifestations, commonly grouped into three principal forms: visceral leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis. The geographic distribution of these forms is highly heterogeneous, with visceral leishmaniasis concentrated predominantly in specific regions of South America, East Africa, and the Indian subcontinent, while cutaneous leishmaniasis shows highest incidence in Latin America, North Africa, and the Middle East (WHO 2022). Transmission dynamics vary according to ecological and epidemiological context. Frequently, leishmaniasis follows zoonotic cycles sustained by a wide range of mammalian reservoirs, including domestic dogs, which frequently develop clinically severe infections, and numerous asymptomatically infected wildlife species covering several taxonomic orders. In other regions,in particular where *L. donovani* is prevalent, transmission is primarily anthroponotic, increasing the public health relevance of human reservoirs (Bourdeau et al. 2020).

The persistence and spread of leishmaniasis are further influenced by environmental modification, population displacement, urban expansion, climatic shifts, and immunosuppression (e.g. HIV co-infection) which collectively contribute to changing epidemiological patterns and expanding areas of endemicity.

2.2 Clinical forms

Leishmaniases manifest in three main clinical forms: cutaneous leishmaniasis (CL), which primarily affects the skin; mucocutaneous leishmaniasis (MCL), involving both skin and mucosal tissues; and visceral leishmaniasis (VL), the most severe form, characterized by systemic infection of internal organs (WHO 2022). The development of the clinical manifestations vary according to the parasite's characteristics and the host's genetic factors, which influence the effectiveness of the immune response (Torres-Guerrero et al. 2017).

Cutaneous leishmaniasis (CL) is the most prevalent clinical manifestation of *Leishmania*l infection. Clinically, it is characterized by the development of cutaneous lesions that typically appear in several weeks to months after the bite of an infected sandfly. Lesions initially present as erythematous papules resembling insect bites and gradually progress to nodular or ulcerative plaques with raised indurated borders and a central crust that may subsequently detach, revealing the ulcer base. Some lesions remain non-ulcerated, persisting as firm nodules. Even in localized CL, patients may exhibit multiple primary lesions - either confined to a single anatomical region or distributed across different sites - often accompanied by satellite lesions, regional lymphadenopathy (occasionally bubonic), or nodular lymphangitis characterized by sporotrichoid-like subcutaneous nodules ('CDC - DPDx - leishmaniasis', 2024).

Two classical clinical patterns are recognized: the "wet" type, commonly associated with *Leishmania major*, characterized by exudative ulcers that resolve within 3-12 months, leaving disfiguring scars; and the "dry" type, mainly due to *Leishmania tropica*, where the crust rarely detaches, healing occurs over more than two years, and recurrences are frequent. *Leishmania infantum* infection may result in atypical presentations resembling eczematous or squamous plaques and can occasionally involve nasal, oral, or laryngeal mucosa, without the destructive features typical of mucocutaneous leishmaniasis.

The incubation period varies according to the infecting species: 2-3 weeks for *L. tropica* and *L. major*, up to 8 months for *L. infantum*, and approximately 1-3 months for most New World species. In some cases, such as infections caused by *Leishmania peruviana* and *Leishmania mexicana*, the disease may develop after 4-10 years, whereas *Leishmania guyanensis* and *Leishmania panamensis* may remain latent for several years before clinical onset. Benign forms of CL typically resolve spontaneously over several months to years, although recurrence may

occur long after apparent healing. Ulcerative lesions frequently result in permanent scarring at the site of infection.

Epidemiologically, the World Health Organization (WHO, 2022) estimates that approximately 95% of CL cases occur in the Americas, the Mediterranean basin, the Middle East, and Central Asia. Globally, CL accounts for between 600,000 and 1 million new cases annually and remains endemic in over 90 countries across tropical, subtropical, and temperate regions (WHO 2022; 'CDC - DPDx - leishmaniasis' 2019).

In the Old World, CL is endemic throughout the Middle East, North Africa, and parts of Asia, where it is mainly caused by *L. major*, *L. tropica*, *L. infantum*, and *L. donovani*. In these regions, the disease is commonly known as "oriental sore", "Baghdad boil," or "Aleppo button" (DermNet®, 2023).

In the New World, CL occurs predominantly in Central and South America and is caused by *L. mexicana*, *L. braziliensis*, and *L. amazonensis*. In these endemic areas, it is often known as "chiclero's ulcer" or "uta," among other regional terms describing its local clinical expression (DermNet®, 2023).

Diffuse cutaneous leishmaniasis (DCL) is a rare manifestation of leishmaniasis characterized by widespread, non-ulcerated nodules and papules on the skin, most prominently affecting the face, trunk, and arms. The condition may exhibit well-demarcated lesions that are typically non-ulcerative, occasionally associated with areas of cutaneous desquamation. DCL can be caused by various *Leishmania* species, including *L. infantum*, *L. aethiopica*, *L. major*, *L. mexicana*, among others. In its early phase, DCL generally presents as a slowly enlarging erythematous macule or plaque that may mimic other dermatologic conditions such as lupus vulgaris or sarcoidosis. Over time - typically after an average period of three years - the disease progresses with the dissemination of multiple plaques and nodules, without mucosal involvement, forming the classic clinical picture of diffuse cutaneous leishmaniasis. This form of the disease is commonly resistant to conventional therapies and may persist, particularly in immunocompromised individuals. Its development is strongly associated with impaired cellular immune responses, allowing uncontrolled proliferation and dissemination of *Leishmania* parasites (Sampaio et al. 2021).

Disseminated leishmaniasis (DL) is an uncommon but severe clinical form of tegumentary leishmaniasis characterized by widespread, polymorphic cutaneous lesions, including inflammatory papules, nodules, and ulcers distributed across multiple body areas. This presentation has been described predominantly in Brazil and is most frequently associated with infection by *Leishmania (Viannia) braziliensis*, although cases have also been documented in the Old World. The pathogenesis of DL reflects a complex interaction between parasite and host factors. *L. braziliensis* is polymorphic, and specific genotypic variants have been linked to different clinical phenotypes and severity of DL. From the host perspective, studies have demonstrated an higher rates of intracellular parasite multiplication in macrophages from DL patients compared with those from individuals with localized CL, suggesting an impaired ability to control infection at the cellular level (Oliveira et al. 2021). As in visceral leishmaniasis, dissemination appears to be associated with defects in T-cell-mediated immunity, although definitive evidence for this mechanism remains limited. Consequently, DL represents a clinically aggressive form of disease with distinct immunopathological characteristics and significant diagnostic and therapeutic challenges (Machado et al. 2024).

Mucocutaneous leishmaniasis (MCL), also known as *espundia*, is one of the most severe forms of *Leishmania* infection. It occurs almost exclusively in the Americas and is caused by species of the *Viannia* subgenus, primarily *Leishmania braziliensis*, *Leishmania guyanensis*, and *Leishmania panamensis* (DermNet® 2023). Unlike CL, which is confined to the skin, MCL is characterized by secondary dissemination of the parasite to mucosal tissues, leading to progressive and often disfiguring destruction of affected areas (Carotenuto et al. 2022; DermNet® 2023).

MCL is generally considered a metastatic complication of cutaneous leishmaniasis (Carotenuto et al. 2022). Mucosal involvement usually occurs after the primary cutaneous lesion has healed, either spontaneously or following local therapy. It may appear months or even years after the initial infection, with an estimated risk of approximately 5% of developing mucocutaneous disease following *L. braziliensis* infection (DermNet® 2023).

More than 90% of MCL cases occur in Bolivia, Brazil, Peru, and Ethiopia (WHO, 2022). Clinically, MCL most commonly presents with ulcerative lesions of the upper aerodigestive tract, particularly involving the nasal septum, oral cavity, oropharynx, and larynx. Early

symptoms may include nasal obstruction, epistaxis, chronic cough, hoarseness, and odynophagia. As the disease progresses, ulceration of mucosal surfaces leads to extensive tissue destruction, which, in the absence of treatment, can result in secondary bacterial infections, sepsis, and severe facial disfigurement (DermNet® 2023).

Mucocutaneous leishmaniasis thus represents a chronic, mutilating, and socially stigmatizing condition with profound functional and psychological consequences. Its delayed onset following primary cutaneous infection underscores the importance of long-term clinical surveillance of patients with CL in endemic regions, particularly those infected with *Viannia* species.

Visceral leishmaniasis **(VL)**, also known as kala-azar or Dumdum fever, is the most severe case of leishmaniasis. According to the World Health Organization (WHO), the disease is fatal in over 95% of untreated cases and is characterized by irregular bouts of fever, progressive weight loss, hepatosplenomegaly and anaemia. Globally, 50 000 to 90 000 new cases of VL are estimated annually, though only 25-45% are officially reported to the WHO. Due to its capacity for outbreaks and high mortality, VL remains a major public health concern (WHO 2022).

VL is endemic primarily in Brazil, East Africa, and India, with additional cases in southern Europe, China, and North Africa. Non-American forms of visceral leishmaniasis are caused by *L. donovani*, *L. infantum*, and *L. tropica*, and are prevalent in India, southern Europe, China, North Africa, and Kenya; whereas, American visceral leishmaniasis, occurring mainly in Central and South America, is caused by *L. infantum* (formerly known as *Leishmania chagasi*) (DermNet® 2023).

The incubation period typically ranges from a few weeks to six months, although in some cases it may extend to several years (Scarpini et al. 2022).

In VL cases caused by *L. donovani* and *L. infantum*, parasites primarily infect the spleen, liver, bone marrow, lymph nodes, and intestinal mucosa, where they replicate extensively within macrophages. The uncontrolled proliferation of parasites in these organs leads to severe hematological alterations, including anaemia, leukocytopenia, and granulocytopenia, due to a shortened lifespan of erythrocytes and leukocytes. Thrombocytopenia may also occur, resulting in hemorrhagic manifestations that further aggravate anaemia (Scarpini et al. 2022).

Moreover, after recovery from VL, most individuals develop a strong and long-lasting cell-mediated immune response, primarily mediated by Th1-type memory T cells that produce

interferon-gamma (IFN-γ), which confers effective and often life-long protection against reinfection (Selvapandiyan et al. 2012; Tiwari et al. 2024; Scarpini et al. 2022).

Post–kala-azar dermal leishmaniasis **(PKDL)** is a muco–cutaneous manifestation that develops in patients after apparent clinical cure of visceral leishmaniasis, particularly when the prior treatment was incomplete, inadequate, or absent. This condition is predominantly endemic in the Indian subcontinent—especially India, Nepal, and Bangladesh—as well as in parts of eastern Africa, where *L. donovani* is the primary etiological agent (Kumar et al. 2021).
The disease may manifest as hypopigmented macules, erythematous papules or plaques, or nodular lesions, most commonly distributed on the face and upper trunk, with possible extension to the extremities, genitalia, and even the oral mucosa, including the tongue. Based on clinical appearance, PKDL is now categorized into macular forms and polymorphic forms, the latter comprising a combination of macules, papules, plaques, and nodules.
Although PKDL does not typically produce significant morbidity in its early stages, it carries substantial epidemiological significance. In fact, individuals with PKDL harbor parasites within their skin and serve as a silent reservoir within the community, particularly in regions where anthroponotic transmission prevails. For this reason, early recognition and active case detection are essential public health measures, forming a critical component of visceral leishmaniasis elimination strategies. Thus, PKDL represents not only a post-treatment dermatologic sequel of visceral leishmaniasis but also a persistent challenge for disease control programs, highlighting the need for clinical vigilance, surveillance, and timely therapeutic intervention (Kumar et al. 2021).

2.3 Pathogenesis of leishmaniases

Aside from the intrinsic characteristics of the *Leishmania* species, the development of leishmaniasis is predominantly determined by the host immune response, which in many contexts exerts a greater influence on disease outcome than parasite-derived factors (Rossi and Fasel 2017a). Following infection, innate immune cells constitute the first line of defense and influence the early immune context. This initial phase establishes conditions that may either promote effective parasite elimination or support its persistence within the host through macrophages activation and regulatory immune pathways. Through antigen presentation and cytokine production, the innate responses subsequently guide the activation and

differentiation of the adaptive immune compartment, in which $CD4^+$ and $CD8^+$ T lymphocytes play essential roles in parasite control but may also drive immunopathology when their activity becomes dysregulated (Lopes et al. 2014).

Protective immunity is often associated with the activation of macrophages and a type 1 response, whereas alternative or regulatory pathways can favor parasite survival and chronicity. The immunological mechanisms engaged during infection vary across *Leishmania* species and anatomical niches, contributing to the diverse clinical spectra of disease (Carvalho et al. 2012).

2.3.1 Innate immune response

The innate immune system is the first line of defense against *Leishmania* infection. Macrophages, natural killer (NK) cells, dendritic cells, and neutrophils shape the local immune environment maynly through cytokine and chemokine production. Macrophages polarize into two main phenotypes: M1 or M2. M1 macrophages, driven by IFN-γ and TNF-α, enhance microbicidal activity via nitric oxide (NO) and reactive oxygen species (ROS). While M2 macrophages, induced by IL-4, IL-13, and IL-10, promote parasite replication through increased arginase activity (Lopes et al. 2014).

Neutrophils are the first cells to arrive at the site of infection. These cells phagocytize pathogens, releasing ROS, and forming neutrophil extracellular traps (NETs). They can also transfer parasites to macrophages, encouraging an anti-inflammatory response (Hurrell et al. 2016; Lopes et al. 2014).

Moreover, dendritic cells present antigens and regulate T cell responses through the production of IL-12 that stimulates Natural Killer (NK) cells to secrete IFN-γ (Rossi and Fasel 2017a).

NK cells contribute early by cytotoxicity and IFN-γ production, regulated by cytokines IL-12, IL-18, and IL-15. Studies suggest that insufficient NK response is correlated with higher parasite loads and disease severity (‘Natural Killer Cells and the Biology of Parasitism’ 2010).

The outcome of infection is thus determined by the coordination and intensity of innate immune responses. In fact, excessive or prolonged inflammation can lead to tissue damage, whereas weak or delayed responses permit parasite persistence.

Overall, innate immunity not only inhibits parasite replication but also establishes the conditions that guide adaptive immunity. The study of these mechanisms is therefore foundamental to understand species-specific disease outcomes.

2.3.2 Adaptative immune response

Adaptive immune responses play a central role in determining the outcome of *Leishmania* infection. Upon antigen presentation by dendritic cells, CD4+ T helper (Th) cells differentiate into Th1 or Th2 subsets. Th1 cells produce key cytokines such as IFN-γ and TNF-α. These cytokines activate macrophages to enhance their microbicidal mechanisms, maynly through nitric oxide production, promoting parasite clearance. A robust Th1 response appears to be strongly associated with resistance to *Leishmania* infection. Conversely, a Th2-biased immune response characterized by IL-4, IL-10, and IL-13 production can suppress macrophage activation, favoring parasite survival and progression of disease. CD8+ T cells contribute to host defense by directly killing infected cells and secreting inflammatory cytokines, supporting the overall cellular immune response.

Immunization studies targeting *Leishmania* antigens, such as nucleoside hydrolase domains, demonstrate that induction of a strong Th1-mediated response with increased IFN-γ production correlates with protective immunity and reduced parasite burden. The balance between these adaptive responses influences protection versus immunopathology, as excessive inflammation or inadequate activation can lead to tissue damage or persistent infection. In summary, effective *Leishmania* immunity relies on a coordinated adaptive response dominated by Th1 cell-mediated macrophage activation and cytotoxic T cell involvement, while Th2 responses can impair control and promote disease (Nico et al. 2010).

2.3.3 Immunopathology across clinical manifestations

Cutaneous leishmaniasis (CL) immunopathology is characterized by a complex interaction between host immune responses and parasite persistence, which together determine disease severity and tissue damage. The Th1 immune response, driven by CD4+ T cells producing interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), activates macrophages to produce ROS and nitric oxide that kill intracellular *Leishmania*. However, excessive or prolonged inflammatory responses, often involving cytotoxic CD8+ T cells, can increase tissue destruction and intensify lesion severity.

On the contrary, Th2 cytokines such as IL-4, IL-10, and transforming growth factor-beta (TGF-β) suppress macrophage activation, promote parasite survival, and contribute to chronic disease. Therefore, regulatory mechanisms and regulatory T cells modulate these responses and influence healing outcomes.

Also neutrophils and NK cells participate in the immune response. Neutrophil extracellular traps contribute to pathogen control but, at the same time, they may promote inflammation. Thus, a successful resolution of CL requires a balanced immune response that clears parasites while limiting tissue damages. This strategy aims to modulate inflammation without compromising immunity (Volpedo et al. 2021).

Mucocutaneous leishmaniasis **(MCL)** is characterized by a chronic and hyperactive inflammatory immune response that leads to significant tissue destruction, particularly in the mucosal areas of the nose and mouth. This exaggerated immunopathology is driven primarily by a robust Th1 response. CD4+ T cells produce interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which activate macrophages to kill the parasites. But, at the same time, this response contributes to tissue damage. In addition, an increased presence of cytotoxic CD8+ T cells intensifies this inflammatory environment, promoting mucosal tissue destruction. Unlike localized CL, MCL exhibits a dysregulated immune profile with reduced regulatory cytokines such as IL-10, which leads to uncontrolled inflammation.
At the same time, cytokines such as IL-17 produced by Th17 cells contribute to neutrophil recruitment and inflammation, intensifying lesion severity. The immunopathogenesis of MCL reflects a delicate balance in which protective immunity against parasites is dominated by tissue damage caused by the immune response. This highlights the critical role of strong cellular immunity and inflammatory mediators in influencing mucocutaneous disease pathology (Volpedo et al. 2021).

Visceral leishmaniasis (VL) immune response reflects a delicate balance between immune activation aimed at parasite elimination and immunoregulatory mechanisms that, when dysregulated, permit parasite survival and contribute to disease severity.
The parasite mainly infects macrophages in the spleen, liver, and bone marrow, leading to systemic disease. Protective immunity is associated with a balance of pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-12, which helps macrophages to kill parasites. Meanwhile, VL progression is strongly influenced by elevated levels of immunosuppressive cytokines, particularly IL-10 and transforming growth factor-beta (TGF-β), which prevent macrophages activation and limit T cell responses. IL-10 plays a central role in disease progression by promoting parasite survival within macrophages and inhibiting the production

of microbicidal molecules like nitric oxide (NO). In patients with active VL, IL-10 expression is elevated in many tissues, which is correlated with reduced parasite clearance. In addition, high antibody titers and immune complex formation may contribute to immunosuppression promoting IL-10 production. The spleen undergoes architectural remodeling, including destruction of marginal zone macrophages, which disrupts immune cell interactions and facilitates parasite persistence. While inflammatory cytokines are elevated, their imbalance combined with immunosuppression leads to reduced granuloma formation in the spleen, while granulomas in the liver remain effective (Kumar and Nylén 2012).

2.4 Diagnosis

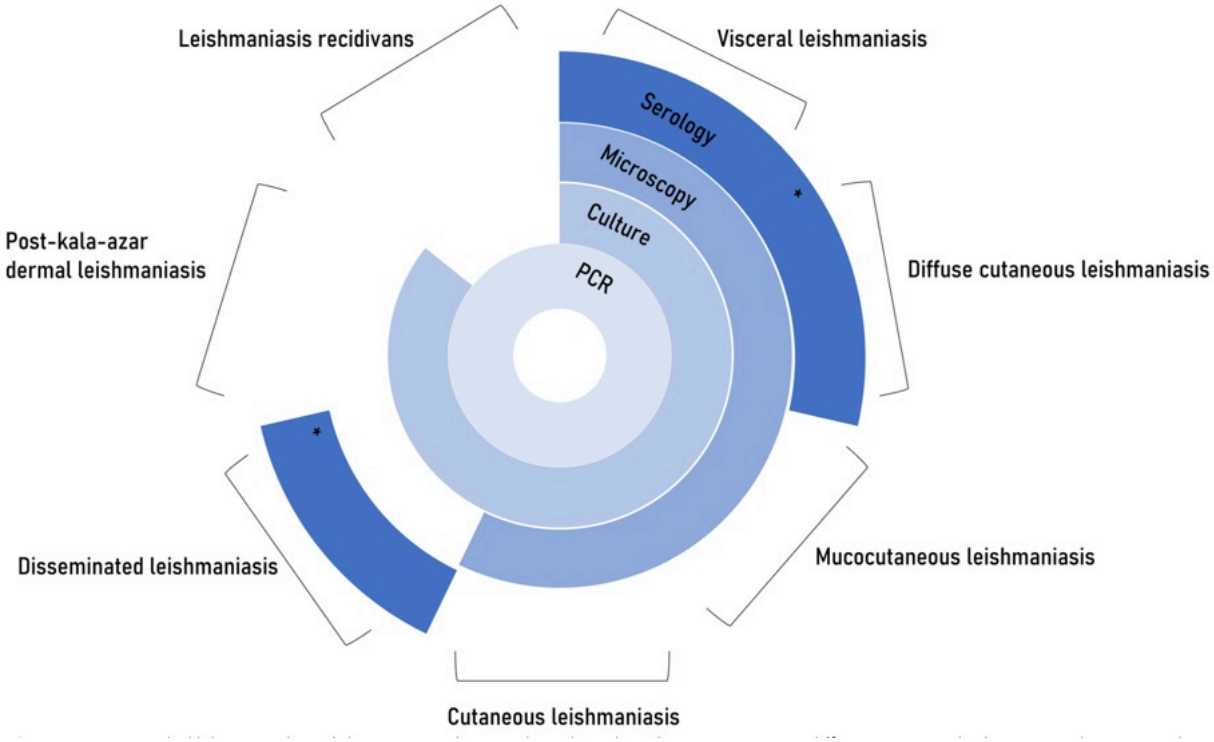

Figure 1 Recommended laboratory-based diagnostic techniques based on clinical presentation. For diffuse cutaneous leishmaniasis, disseminated leishmaniasis and leishmaniasis recidivans, where recommendations are not available from a public health organisation or reference laboratory, guidance is taken from relevant studies [43–50]. The asterisk indicates the rK39 rapid immunochromatographic test kit only. PCR Polymerase chain reaction. From Gow et al. 2022.

Accurate diagnosis of leishmaniasis is essential for effective clinical management and epidemiological control. The disease shows the wide range of clinical manifestations and its symptoms often overlap with other infectious diseases. Diagnostic approaches are generally

classified into direct and indirect methods, depending on whether they target the parasite itself or the host immune response (Figure 1).

Indirect diagnosis aims to detect the host's immunological response to *Leishmania* infection. This is primarily achieved through serological testing, with indirect immunofluorescence considered the gold standard. Enzyme-linked immunosorbent assays (ELISA) are also widely employed to detect and quantify specific anti-*Leishmania* antibodies. These tests are particularly useful for visceral leishmaniasis, even if their sensitivity may be limited in cutaneous forms or in immunocompromised individuals.

Direct diagnosis involves the identification of the parasite or its genetic material in biological specimens. Techniques include microscopic examination of tissue smears, cell culturing, and molecular methods such as polymerase chain reaction (PCR). The latter is highly sensitive and allows species-specific detection. Direct diagnostic methods are crucial for confirming active infection, particularly in cases with low antibody titers or atypical clinical presentations.

In general, an integrated approach combining clinical evaluation with both direct and indirect laboratory methods provides the highest accuracy in diagnosing leishmaniasis and guiding appropriate treatment strategies.

2.4.1 Microscopy

For microscopic detection, direct smears from tissue aspirates are typically prepared and stained to visualize the parasite. In these preparations, *Leishmania* amastigotes appear as small, round structures measuring approximately 2-4 µm in diameter. Promastigotes grown in culture are elongated, ellipsoid to slender, and range from 15 to 25 µm in length. Staining helps cellular and parasites observation, with Giemsa and Leishman stains as the most frequently used (Barcia 2007). Once stained, amastigotes are usually observed inside macrophages, with a pale blue cytoplasm, a red nucleus, and a purple-pink kinetoplast. The parasitic burden can be quantified using the modified Ridley's parasitic index, which estimates the number of amastigotes present in the sample (Ejazi et al. 2018).

Microscopic detection sensitivity varies from 54% to 96%, while specificity can be as low as 46%. This depends on the type of sample collected, the quality of staining reagents, and the technical expertise of the operator (Mukhtar et al. 2018).

The stage of infection further influences sensitivity: in cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL), amastigote numbers tend to decrease as the infection

progresses, whereas in visceral leishmaniasis (VL), parasite load increases during chronic stages. In VL, the highest sensitivity is generally achieved using more invasive specimens, such as splenic aspirates.

Microscopy observation can confirm *Leishmania* presence at the genus level, but can't distinguish species, as their morphology is similar (Gow et al. 2022).

2.4.2 Cell culturing

Cell culture represents a valuable approach to enhance the sensitivity of *Leishmania* detection. Various culture media are employed for parasite isolation and growth. These include Tobie's medium, Schneider's *Drosophila* medium, Senekjie's medium, Medium 199, RPMI 1640, Grace's insect medium, brain-heart infusion medium, blood agar (with rabbit blood), and chocolate agar. Each medium supports parasite growth differently. For instance, Tobie's medium promotes the transformation of amastigotes into promastigotes, while Grace's medium is particularly effective in increasing overall cell density (Gow et al. 2022). An innovative approach in cell culturing is the microcapillary culture method, in which the sample is concentrated within capillary tubes, which increases sensitivity from 69.2% to 92.3% (Allahverdiyev et al. 2005). However, culture-based methods generally require several days to weeks to yield definitive results.

2.4.3 Histology

In CL, histological analysis typically involves the examination of tissue sections 4-5 µm thick. Sections are stained with Giemsa or hematoxylin and eosin to visualize histiocytes containing intracellular amastigotes, which are often located near the epidermal layer. This method works better in early stages. In advanced lesions, amastigotes may be few or undetectable. Thus, histology is considered one of the least sensitive diagnostic approaches, with reported sensitivities ranging from 42% to 70%, and its specificity can reach 100% (Aronson and Joya 2019; Ranawaka et al. 2012; Daneshbod et al. 2011). A further limitation is that *Leishmania* amastigotes may be confused with other intracellular organisms, including *Toxoplasma gondii*, *Mycobacterium leprae*, fungi (e.g., *Histoplasma*), or even tissue artifacts, which may require the use of alternative stains for accurate differentiation (Daneshbod et al. 2011).

Histological examination is not commonly applied in visceral leishmaniasis (VL), where clusters of histiocytes, occasional amastigotes, or morphological changes may be observed. Additionally, amastigotes are often distributed non-uniformly across tissue sections of varying thickness. This can make the analysis time-consuming and reduce the sensitivity (Gow et al. 2022).

2.4.4 Serological tests

The Enzyme-Linked Immunosorbent Assay (ELISA) is widely employed in both endemic and non-endemic regions for the detection of the host antibody response to *Leishmania* infection. The sensitivity of the ELISA assay depends largely on the nature of the antigen used to bind specific antibodies. For instance, when crude soluble antigens (CSA) are used, the assay can achieve sensitivities ranging from 80% to 100% (Gow et al. 2022; Maia et al. 2012). However, several limitations should be considered. Early in infection, the antigen-specific antibody levels may be insufficient for reliable detection. Antibodies can alsopersist long after successful treatment, making difficult the distinction between active, resolved or relapsed disease. Moreover, ELISA accuracy decreases in immunocompromised individuals, and cross-reactivity with other infections prevalent in leishmaniasis-endemic regions, such as Chagas disease, may lead to false-positive results (Kumar et al. 2020).

2.4.5 Molecular detection

DNA-based detection of *Leishmania* employs a wide range of genomic targets whose sensitivity is largely determined by the copy number of each target within the parasite's genome (Gow et al. 2022). Molecular methods have represent a new phase in clinical diagnostics for leishmaniasis, by offering improved sensitivity, specificity, speed, user-friendliness and suitability for deployment in endemic regions. Approaches such as real-time PCR can additionally provide quantitative information on parasite burden and treatment response, surpassing the capabilities of conventional diagnostic tools. They also enable the detection of asymptomatic patients or individuals infected prior to the appearance of clinical signs, which is an aspect of major relevance for disease surveillance and blood donor screening.

The type of molecular target examined varies according to the clinical form of leishmaniasis. In cases of post-kala-azar dermal leishmaniasis, split-skin smears and biopsy specimens are the

procedures most frequently implemented (Adams et al. 2013). For visceral leishmaniasis (VL), splenic aspiration, as well as bone marrow or lymph node sampling, are routinely performed, with diagnostic sensitivity increasing in proportion to the invasiveness of the procedure: 93–99% for spleen, 53–86% for bone marrow, and 53–65% for lymph (de Ruiter et al. 2014).

Polymerase chain reaction (PCR) is increasingly used for the detection of *Leishmania* spp. Several commercially available kits rely on PCR-based approaches to detect members of the genus *Leishmania*, targeting markers such as 18S rRNA, ITS1 and IT2 loci, *cytb*, 18S ribosomal sequences, the GP63 protein, and kDNA minicircles. Among the various PCR methodologies, conventional PCR (cPCR), real-time PCR (q-PCR), and droplet digital PCR (ddPCR) are the most widely applied(Gow et al. 2022).

A sensitivity and specificity of 100% have been achieved with cPCR when applied to lesion samples (Nateghi Rostami et al. 2020). In cases where lower sensitivity and specificity are observed for certain specimen types, nested PCR is employed. In this approach, an initial PCR is performed to amplify the target gene, followed by a second amplification using the first-round amplicons. This method has been recently described for the detection of *Leishmania* spp. (Deepachandi et al. 2019).

Real-time PCR (qPCR) is a probe-based technique that provides higher sensitivity and specificity than conventional PCR (93.9% sensitivity; 100% specificity). It is commonly applied to peripheral buffy coat samples from patients with visceral leishmaniasis (VL).

A recently developed approach is droplet digital PCR (ddPCR), a probe-based technique that enables the absolute quantification of target DNA molecules. One of the most cited studies is that of Ramírez et al., who developed a ddPCR assay targeting 18S rDNA that is capable of detecting seven *Leishmania* species (Ramírez et al. 2019).

2.5 Treatment

The choice of treatment depends on several factors, including geographical areas, concomitant pathologies and the parasite species (Frézard et al. 2022). Therapeutic approaches often depend on *Leishmania* species present in a specific geographic area. For instance, data from clinical trials of VL therapy in India may not apply to VL caused by *L. donovani* in other regions, to infections caused by different *Leishmania* species, or to the treatment of cutaneous (CL) and mucocutaneous leishmaniasis (MCL) (CDC 2024a).

Currently, the only FDA-approved treatments for leishmaniasis are liposomal amphotericin B and miltefosine (CDC 2024a). Liposomal amphotericin B (AmBisome®) is primarily used for the treatment of VL. It is the standard therapy for immunocompetent patients in the Mediterranean region and also commonly administered to individuals with *Leishmania*–HIV co-infection (Sundar and Chakravarty 2010). Miltefosine is an orally administered drug used to treat the visceral and mucosal forms of leishmaniasis. Its use is restricted to patients aged 12 years and older who are infected with one of three species within the *Leishmania (Viannia)* subgenus (*L. braziliensis*, *L. panamensis*, and *L. guyanensis*) (Astman et al. 2024).

In general, one of the earliest signs that treatment is effective is a gradual reduction in induration, as the lesion slowly flattens. Healing, especially in large ulcerative lesions, often progresses even after the course of therapy has ended, with tissue repair continuing over time. However, when relapse occurs, the first indication of clinical reactivation typically appears at the outer edge of the lesion, where new activity becomes noticeable before spreading more widely (CDC 2024a).

In addition to the currently approved drugs, several other anti-*Leishmania*l therapies remain in clinical use, including pentavalent antimonials, paromomycin, azole derivatives, and pentamidine, although their success rates vary according to parasite species, anatomical site of infection, and duration of follow-up (van Griensven et al. 2024). Clinical improvement, such as wound re-epithelialization or splenomegaly reduction, does not always indicate parasitological cure, as viable *Leishmania* have been isolated from scar tissue and lymph nodes after apparent remission (van Griensven et al. 2024).

2.6 Prevention

Given that no vaccines are currently available to prevent leishmaniasis infection, preventive strategies necessarily focus on vector control and personal protective measures. Protection against the sand fly vector includes the application of residual insecticides and the use of physical barriers such as bed nets and curtains, with a preference for insecticide-treated materials when possible. Insecticides may be applied to bed nets, clothing, and household textiles to further reduce the likelihood of vector contact (CDC 2024b).

In addition, minimizing exposed skin is recommended to limit opportunities for sand fly blood feeding. This can be achieved by wearing long-sleeved shirts, long trousers, and socks, particularly during peak vector activity periods. The application of EPA-registered insect

repellents to exposed skin and clothing provides an additional layer of protection; formulations containing DEET are generally considered the most effective. Collectively, these measures form the basis of current evidence-based approaches to reducing individual risk in endemic settings (CDC 2024b).

In dogs, leishmaniasis prevention should be implemented through a multimodal strategy that combines the use of repellents with vaccination. Topical insecticidal repellents are effective during periods of sand fly activity and help reduce the likelihood of vector contact and subsequent *Leishmania* transmission ('LEISHVET FACT SHEET', n.d.). Furthermore, numerous studies have demonstrated that vaccination can significantly decrease the risk of clinical disease development, supporting its role as a complementary preventive measure within an integrated control program (Zini et al. 2020).

To prevent canine leishmaniasis (CanL) effectively, topical insecticides should be administered to dogs residing in (or travelling to) endemic regions. This should be maintained throughout the entire period of potential exposure to sand flies. Permethrin-based spot-on preparations repel sand flies and prevent feeding for approximately three to four weeks. For dogs travelling to endemic areas, the product should be applied at least two days before potential exposure. Insecticidal collars represent an alternative long-acting preventive measure. Deltamethrin-impregnated collars can protect against sand fly bites for prolonged periods, in some cases up to twelve months depending on the product. Collars with flumethrin and imidacloprid have also been shown to repel sand flies and, in clinical field studies, have reduced the risk of *L. infantum* infection for up to eight months. All collar types should be applied in advance of exposure, ideally one to two weeks before dogs enter areas where transmission may occur ('LEISHVET FACT SHEET', n.d.).

In Europe, the only vaccine currently available is Letifend® (Lentifarma Laboratories), which contains the recombinant Protein Q composed of five distinct *L. infantum* antigens, for use in healthy seronegative dogs. In addition, in 2023 the European Medicines Agency authorized a new DNA-based vaccine formulated with a plasmid encoding the *L. infantum* LACK antigen (*Leishmania* analogue of the receptor for activated protein kinase C), further advancing ipreventive immunization strategies ('LEISHVET FACT SHEET', n.d.).

3 *Leishmania* spp.

3.1 Taxonomic classification of *Leishmania*

The genus *Leishmania* comprises protozoan parasites belonging to the order *Kinetoplastida* and the family *Trypanosomatidae*. Traditionally, it is taxonomically divided into four subgenera (*Leishmania*, *Viannia*, *Sauroleishmania* and *Mundinia*) which are distinguished by their host specificity, geographical distribution, and evolutionary history (Figure 1) (Klatt et al. 2019; Akhoundi et al. 2016).

To date, approximately 53 species of *Leishmania* have been described, 31 of which infect mammals and 20 are pathogenic to humans (Akhoundi et al. 2016). Among these recognized species, 29 occur in the Old World, 20 in the New World, three are distributed across both regions, and one species (*L. macropodum*) is found exclusively in Australia (Klatt et al. 2019).

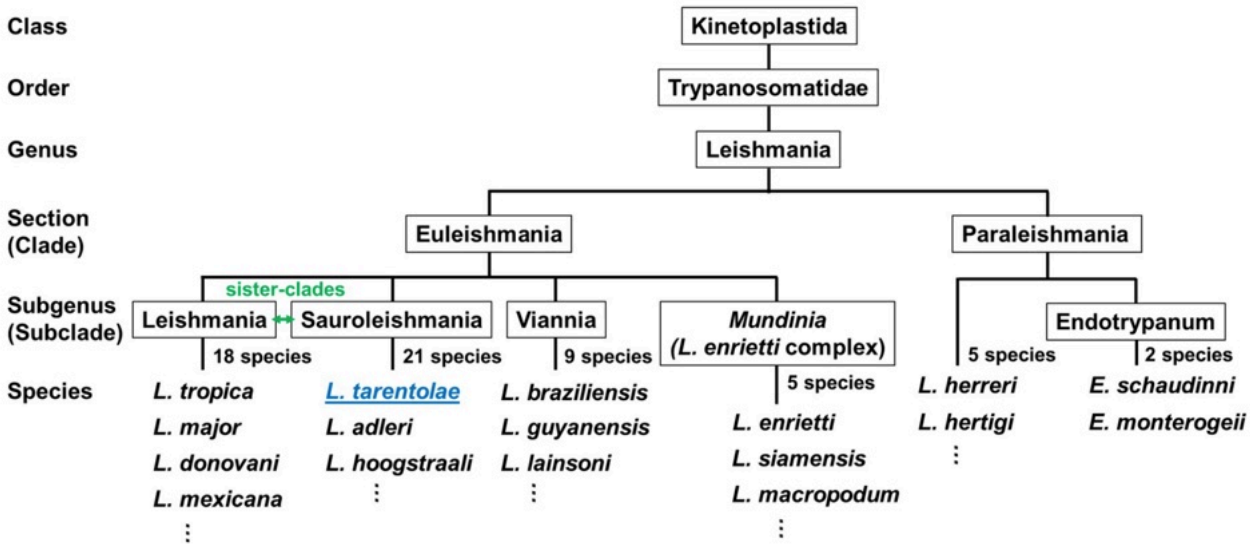

Figure 2 Phylogeny-based classifcation of the genus *Leishmania* (Klatt et al. 2019).

- The **subgenus *Leishmania* (Leishmania)** occurs in both the Old and New Worlds, incuding Central and South America, Southern Europe, Asia, and Africa (Klatt et al. 2019). Species belonging to this subgenus, such as *L. donovani*, *L. infantum*, and *L. major*, are responsible for both cutaneous and visceral forms of leishmaniasis in mammals, including humans. Transmission is primarily mediated by sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World, with additional phlebotomine genera involved in some transmission cycles (Bates 2007) .

- The **subgenus *Leishmania* (Viannia**) is confined to tropical regions (Central and South America), where it parasitizes mammals, including humans. This parasites comprises several species distributed across the Neotropics, notably *L. braziliensis*, *L. guyanensis*, *L. panamensis*, and *L. peruviana*. Transmission occurs through sand flies belonging to the genus *Lutzomyia*. These species are primarily responsible for cutaneous leishmaniasis; however, in some cases, the parasites may disseminate to the nasopharyngeal mucosa, causing mucocutaneous leishmaniasis (Llanes et al. 2015).

- The **subgenus *Leishmania* (Sauroleishmania)** includes 21 species, all restricted to the Old World. These parasites are transmitted by sand flies of the genus *Sergentomyia* and primarily infect reptiles, particularly lizards. Representative species include *L. tarentolae*, *L. hoogstrali*, *L. adleri*, and *L. gymnodactyli* (Klatt et al. 2019).

- The **subgenus *Leishmania* (Mundinia)** was established in 2018 and currently comprises five recognized species. Among these, *L. martiniquensis*, *L. orientalis*, and *L. chancei* are known to infect humans, while *L. enriettii* infects guinea pigs and *L. macropodum* has been isolated from marsupials. Geographically, *Mundinia* is present in Asia, Africa, the Americas, and Australia. Unlike other *Leishmania* subgenera, which are transmitted by phlebotomine sand flies, species of *Mundinia* appear to be primarily transmitted by biting midges of the genus *Culicoides*, suggesting a distinct vector–parasite adaptation within the genus (Vojtkova 2021).

3.2 Geographical origin of *Leishmania* species

The occurrence of *Leishmania*-like species has been documented since prehistoric times through fossil evidence preserved in amber. The earliest known record was identified within the alimentary tract of the extinct sand fly *Palaeomyia burmitis*, preserved in Cretaceous Burmese amber dating to approximately 100 million years ago. This *Leishmania*-like organism was subsequently assigned to a newly established collective fossil genus, *PaleoLeishmania*, and described as *PaleoLeishmania proterus* (Steverding 2017). A second *Leishmania*-like fossil was later discovered in the extinct sand fly *Lutzomyia adiketis*, preserved in Dominican amber from

the Miocene epoch (20–30 million years ago), and subsequently described as *PaleoLeishmania neotropicum (Steverding 2017)*.
This fossil evidence reveals a digenetic life cycle in *Leishmania* and suggest that sand flies acquired the parasites through blood feeding. This finding indicates that these insects served as vectors of *Leishmania*-like protozoa from the mid-Cretaceous to the early-Miocene (Steverding 2017).

Phylogenetic and biogeographical evidence suggests that *Leishmania* evolved during the Mesozoic era (252–66 MYA), preceding the disintegration of Pangaea (Thomaz-Soccol et al. 1993). However, the origin and biogeographical distribution of the genus *Leishmania* have long been subjects of debate, reflecting the interaction between parasite evolution and the geological and ecological history of their vertebrate hosts and sand fly vectors. Three major hypotheses have been proposed to explain the current global distribution of *Leishmania*: the Gondwanan hypothesis, the Palaearctic hypothesis and the Neotropical hypothesis (Steverding 2017)**.**

The **Gondwanan hypothesis (or supercontinent hypothesis)** propose that *Leishmania* originated before the breakup of the supercontinent Gondwana, more than 100 million years ago. According to this view, the diversification of the parasite occurred while the continental masses were still united, and the subsequent fragmentation of Gondwana led to the geographic isolation and independent evolution of different *Leishmania* lineages. Building on this view, Momen and Cupolillo suggest that following the breakup of Gondwana during the Mesozoic, the subgenera *Leishmania* and *Sauroleishmania* evolved in Africa, whereas the subgenus Viannia diverged in South America (Momen and Cupolillo 2000).
This interpretation views the present distribution of *Leishmania* species across Africa, South America, and Asia as a result of vicariant events rather than later dispersal. Molecular phylogenetic evidence, which indicates deep divergence between major subgenera such as *Leishmania (Leishmania)* and *Leishmania (Viannia)*, provides partial support for this scenario (Steverding 2017).

The **Palaearctic hypothesis,** first proposed by Lysenko in 1971, suggests that *Leishmania* originated in the Palaearctic region—an area that during the Paleocene epoch (66–56 million

years ago) encompassed Europe, Asia north of the Himalayas, northern Arabia, and North Africa north of the Sahara—followed by a subsequent dispersal to the New World. According to this view, the present distribution of *Leishmania* primarily reflects biological dispersal events that occurred after continental drift, likely facilitated by host migrations and the expansion of sandfly vector ranges(Lysenko 1971).

The **Neotropical hypothesis** proposes that the genus *Leishmania* originated in the Neotropical region. This view is based expecially on the observation that *Leishmania* species exhibit greater diversity in the New World than in the Old World, which has been interpreted as evidence of a Neotropical origin. However, this interpretation conflicts with several established scientific findings, suggesting instead that the high species diversity in the Neotropics may result from an accelerated rate of evolution driven by climatic fluctuations, expansion of host range, and geographical isolation (Steverding 2017).

Overall, the contrasting Gondwanan and Palaearctic–Neotropical hypotheses reflect two complementary perspectives on *Leishmania* biogeography: one emphasizing ancient vicariance linked to continental drift, and the other highlighting more recent ecological and evolutionary processes associated with host–vector coevolution.

3.3 *Leishmania* spp. life cycle

The *Leishmania* parasite exhibits two distinct morphotypes: the promastigote form, which occurs in the sand fly vector, and the amastigote form, which develops within the vertebrate host.

The **amastigotes** represent the intracellular, non-flagellated form of the *Leishmania* parasite found within the vertebrate host. Specifically, they reside in the phagolysosomes of macrophages, where they can survive the acidic and enzymatic environment and replicate successfully. Morphologically, amastigotes are spherical or ovoid and smaller than the promastigote form, measuring approximately 2–5 µm in length (Besteiro et al. 2007).

The **promastigote** form is the extracellular, flagellated stage of *Leishmania* found in the sand fly vector. It is an elongated cell with a single flagellum that enables active motility (Besteiro et al. 2007). Within the sand fly's alimentary tract, promastigotes multiply and undergo several morphological transitions, including elongated nectomonads, short nectomonads, metacyclic

promastigotes, rounded promastigotes, and haptomonads (Sunter and Gull 2017; Ticha et al. 2021). The metacyclic promastigote represents the infective form transmitted to the vertebrate host during the sand fly's blood meal. Once inoculated into the host's skin, promastigotes are rapidly internalized by macrophages, initiating the intracellular stage of infection.

3.3.1 *Leishmania* life cycle in the insect vector

The development of *Leishmania* parasites within the sand fly vector is confined to the insect's digestive tract (Cecílio et al. 2022).

The sand fly gut is anatomically divided into three main regions:

- Hindgut**:** the posterior section of the digestive tract, extending from the pylorus to the rectum.
- Midgut**:** the central region, located between the cardia and the pylorus, encompassing both thoracic and abdominal portions.
- Foregut**:** the anterior section, extending from the mouth to the stomodeal valve situated within the cardia.

The life cycle of *Leishmania* within the sand fly vector can be classified according to the localization of parasite proliferation and differentiation along the digestive tract (Figure 3):

- Suprapylarian cycle**:** parasite development is restricted to the midgut. This type of cycle is the most common among *Leishmania* species pathogenic to humans. Representative species include *Leishmania (Leishmania) major*, *L. tropica*, *L. donovani*, *L. infantum*, *L. mexicana*, and *L. amazonensis*.
- Peripylarian cycle: characteristic of species belonging to the subgenus *Viannia*, in which parasites initially colonize the hindgut before migrating anteriorly to the midgut. Representative species include *Leishmania (Viannia) braziliensis*, *L. panamensis*, *L. guyanensis*, *L. peruviana*, *L. lainsoni*, and *L. shawi*.
- Hypopylarian cycle: in this case, parasite development occurs predominantly within the hindgut, with little or no migration toward the anterior regions of the gut. This type of cycle is typical of *Leishmania*-like parasites infecting reptiles or small mammals. Representative species include *Leishmania (Sauroleishmania) tarentolae*, *L. adleri*, *L. hoogstraali*, and *L. gymnodactyli*.

After ingestion of an infected blood meal, *Leishmania* amastigotes differentiate into **procyclic promastigotes** within the hindgut of the sand fly. This transformation occurs rapidly as a consequence of environmental changes, including a decrease in temperature and an increase in pH (Dostálová and Volf 2012; Lawyer et al. 1990). Procyclic promastigotes subsequently replicate in the hindgut. In the midgut, the blood meal is enclosed by a type I peritrophic membrane, which offers a semi-protective function-preserving the microvilli from digestive enzymes, mitigating the potentially deleterious effects of blood digestion products and providing a barrier against pathogens. Consequently, within approximately 48 hours, procyclic promastigotes reduce their replication rate and differentiate into **nectomonad promastigotes** (Rogers 2012). These elongated, motile forms are capable of migrating from the peritrophic membrane into the midgut lumen, where they attach to the gut epithelium. This attachment is essential to prevent their elimination during the sand fly's defecation (Cecílio et al. 2022).

Subsequently, nectomonad promastigotes migrate to the anterior midgut, where they differentiate into **leptomonad promastigotes**. At this stage, the promastigotes replicate within the midgut and then transform into **metacyclic promastigotes**, the highly motile and infective stage for vertebrate hosts. Alternatively, some leptomonad promastigotes attach to the stomodeal valve and differentiate into **haptomonad promastigotes**, but the functional role of this form remains unclear (Cecílio et al. 2022).

In addition, a further developmental stage, known as the **retroleptomonad**, may arise through the de-differentiation of metacyclic promastigotes after the intake of a second blood meal, when new nutrients become available. Retroleptomonads resemble leptomonads morphologically and retain the capacity for replication. Once stress conditions subside, typically after defecation of the second blood meal, retroleptomonads re-differentiate into metacyclic promastigotes. The replication of retroleptomonads contributes to enhanced vector competence, resulting in an higher parasite load and a more homogeneous population of metacyclic forms (Cecílio et al. 2022).

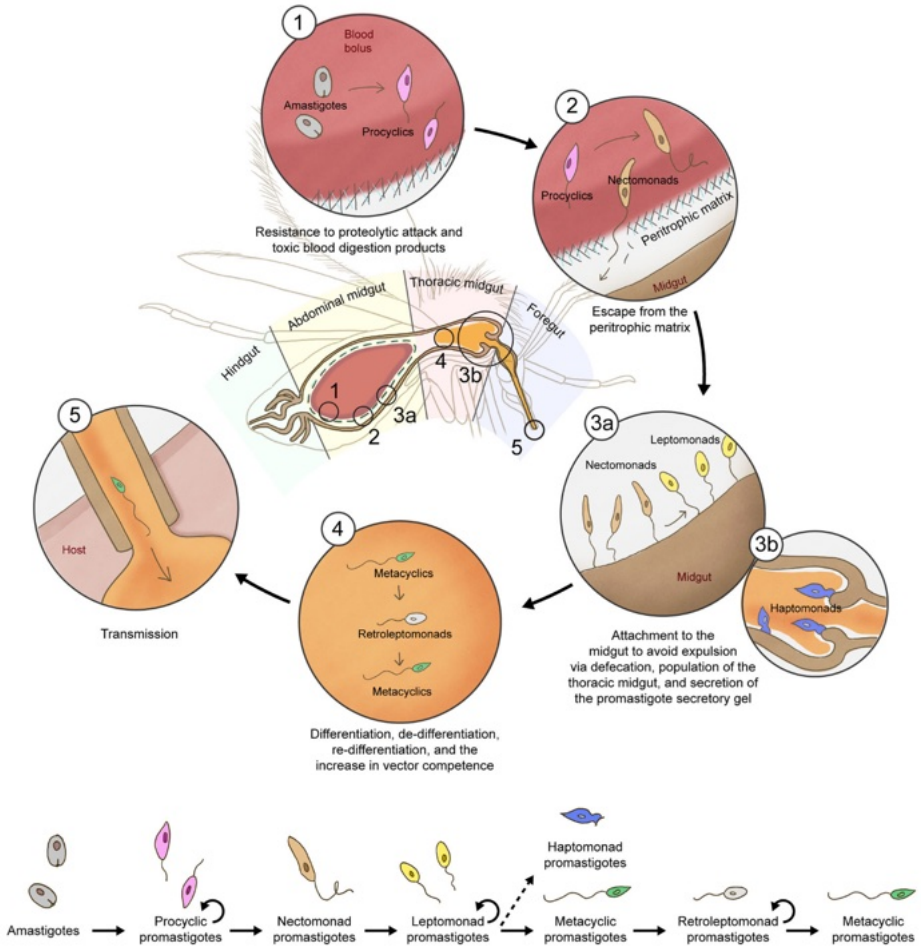

Figura 3 *Leishmania* development within the sand fly midgut (Cecílio et al. 2022). Schematic representation of the different forms of *Leishmania* parasites within the sand fly vector and of the major barriers they must overcome to establish a productive infection—including the resistance to proteolytic attack/toxic byproducts of the digestion of blood (1), "escape" from the perothrophic matrix (2), attachment to the midgut to avoid expulsion (3a), attachment to (and impairment of) the stomodeal valve (3b), and (de-)differentiation and replication dynamics (4)—and ensure their transmission (5) to a new host. A linear life cycle with the different parasite forms within the vector is also represented; the circular arrows highlight the replicative parasite forms.

3.3.2 *Leishmania* life cycle in the vertebrate host

During the blood meal of an infected sand fly, metacyclic promastigote forms of *Leishmania* are inoculated into the skin of the vertebrate host (Figure 4). These forms - the infective stage of the parasite - are adapted to survive in the extracellular environment immediately after transmission (Clos et al. 2022). In response to the vector's bite, an intense recruitment of innate immune cells occurs, particularly polymorphonuclear neutrophils, which constitute the first line of host defense and the first cells to interact with the promastigotes (Peters et al. 2008).

Once phagocytosed by neutrophils, the parasites begin their transformation into the intracellular amastigote stage, which is metabolically adapted to survival within the phagolysosomal compartment by evading the antimicrobial mechanisms. In particular,

Leishmania actively moodulates the host immune sistem by interfering with the host cell signal transduction pathways (Clos et al. 2022).

The transformation into amastigotes takes place before the neutrophils undergo apoptotic death. The apoptotic bodies derived from these infected neutrophils are subsequently engulfed by resident or recruited macrophages at the site of infection, in a process referred to as the “Trojan horse” mechanism, which allows the parasite to enter its target cells while evading immediate immune activation (Chaves et al. 2020; Carlsen et al. 2015).

Within macrophages, amastigotes multiply by binary fission inside acidic intracellular vacuoles enriched in lysosomal markers. These compartments provide a favorable environment for parasite survival, as *Leishmania* species possess efficient mechanisms to resist host-derived reactive oxygen and nitrogen species. As infection progresses, the number of amastigotes per infected cell increases substantially, ultimately leading to macrophage lysis and the release of parasites that can infect new phagocytic cells (Courret et al. 2002).

Macrophages are the principal cell type found infected in host tissues; however, other immune cells, such as dendritic cells and neutrophils, have also been identified as potential reservoirs or transient carriers of the parasite. This multicellular tropism highlights the ability of *Leishmania spp.* to modulate both innate and adaptive immune responses, promoting chronic infection persistence and tissue dissemination (Silva-Moreira et al., n.d.).

The cycle of *Leishmania* completes as infected macrophages harboring amastigotes are ingested by sand fly vectors during a blood meal; within the vector, the parasites differentiate into promastigotes, multiply, and migrate to the proboscis, ready to infect a new vertebrate host.

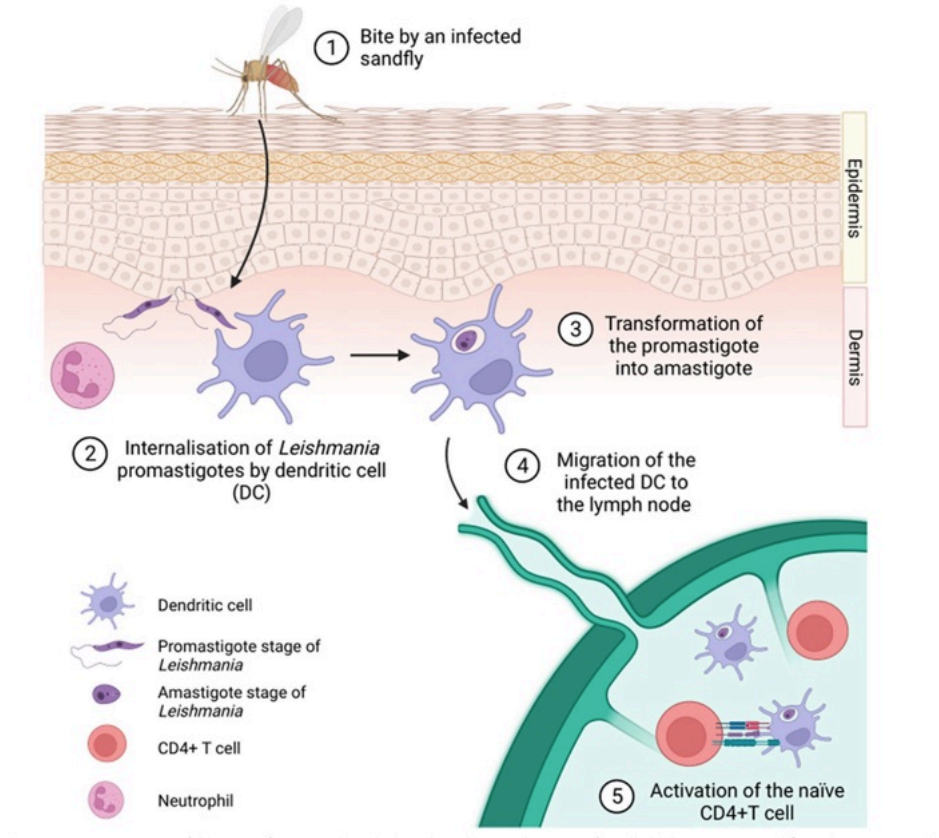

Figura 4 Schematic representation of the cutis of a mammal and a lymph node. A *Leishmania*-infected Phlebotominae sand fly is also represented (1). A dendritic cell (DC) is represented during the process of phagocytosis of a *Leishmania* promastigote (2), the transformation of the promastigote into the amastigote stage (3) and then the migration of the infected DC towards the lymph node (4). Inside the lymph node, DCs present the antigens to CD4+T cells. A neutrophil is also shown as these cells play a role in the first phases of mammalian infection by *Leishmania* spp (5). From Bandi et al. 2023.

3.3.2.1 *Leishmania* and the host immune response

During the early phase of infection, *Leishmania* parasites exploit the pro-inflammatory properties of sand fly saliva, which plays a crucial role in recruiting phagocytes to the bite site and thereby representing a key determinant of infection outcome (Rossi and Fasel 2017b). Once internalized by macrophages, *Leishmania* establishes a long-lasting infection by residing within the phagolysosome, an organelle characterized by low pH and an abundance of lytic enzymes. In the first hours following entry, macrophages mount an effective anti-*Leishmania* response centered on the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide (O_2^-) and nitric oxide (NO), produced through enzymatic pathways activated by phagocytosis. However, *Leishmania* parasites evolved sophisticated mechanisms to counteract this oxidative burst. Notably, they can secrete or induce host macrophages to express arginase, an enzyme that competes with inducible nitric oxide

synthase (iNOS) for the amino acid arginine. This diversion of arginine metabolism serves a dual purpose: it provides the parasite with essential nutrients, such as L-ornithine for polyamine synthesis, and markedly reduces the production of parasitotoxic NO, thereby promoting intracellular survival. Through these strategies, *Leishmania* actively shapes and withstands oxidative stress from the moment of entry onward.

As the infection progresses, antigen-presenting cells such as dendritic cells migrate to secondary lymphoid organs, where they prime naïve T lymphocytes. The clinical outcome of leishmaniasis depends on the nature of this response. $CD4^+$ T cells can differentiate into distinct effector subsets, primarily Th1 and Th2 cells, which exert opposing influences on disease progression. A robust Th1 response—characterized by cytokines such as IFN-γ and IL-12—drives macrophage activation, enhances microbicidal functions, and is associated with effective control of the parasite. In contrast, a Th2-skewed response, dominated by cytokines such as IL-4 and IL-10, promotes parasite persistence and contributes to disease development. $CD8^+$ T cells also participate in host defense by producing cytotoxic molecules and pro-inflammatory cytokines, although their contribution varies with the *Leishmania* species and tissue context. Overall, the dynamic interplay between innate defenses, T-cell polarization, and cytokine networks ultimately determines whether the host achieves parasite control or progresses toward pathology (Rossi and Fasel 2017b).

4 *Leishmania tarentolae*

Leishmania tarentolae is a non-pathogenic member of the subgenus *Sauroleishmania*. Indeed, the 21 species belonging to this subgenus have been studied, as they are primarily associated with reptiles (families *Agamidae*, *Gekkonidae*, *Lacertidae*, *Scincidae*, and *Varanidae*) and are generally considered non-infective to mammals (Bandi et al. 2023; Ticha et al. 2021).

Originally isolated from *Tarentolae mauritanica*, *L. tarentolae* is the most studied *Sauroleishmania* species owing to its non-pathogenic nature and close phylogenetic relationship to mammalian-infective *Leishmania* species (Ticha et al. 2021).

This species is restricted to the Old World, particularly in Europe, North Africa, and the Middle East, and is generally transmitted by sand flies belonging to the genus *Sergentomyia* (Mendoza-Roldan, Votýpka, et al. 2022; Bandi et al. 2023). Sand flies of this genus are known to feed primarily on reptiles. However, previous studies have reported that some *Sergentomyia* species may also feed on mammals, including humans (Maia and Depaquit 2016; Mendoza et al. 2021;

Senghor et al. 2016; Alvaro et al. 2025). This raises the question of whether these insects could potentially act as vectors for *Leishmania* species pathogenic to mammals. In fact, members of the genus Sergentomyia have been found to host mammal-pathogenic species such as *L. infantum*, suggesting possible cross-transmission potential. Moreover, *L. tarentolae* has been detected in sand fly species that usually feed on mammals, such as *P. perniciosus* and *P. perfiliewi*, and molecular evidence has also revealed its presence in mammal hosts (Mendoza et al. 2021; Pombi et al. 2020). Furthermore, *L. infantum* has been identified in reptilian hosts, indicating that host specificity and vector–parasite relationships may be considerably more complex and overlapping than previously assumed (Mendoza-Roldan et al. 2022).

4.1 *Leishmania tarentolae* life cycle

During the blood meal of an infected sand fly, *L. tarentolae* promastigotes are transmitted to the reptile host. The life cycle of *L. tarentolae* remains only partially characterized and it probably differs from that of *Leishmania* species pathogenic to mammals. Studies suggest that a subset of promastigotes are phagocytosed by the host immune cells, such as macrophages and monocytes, where they differentiate into amastigote-like forms. Accordingly, in blood smears, promastigote-like forms are typically observed extracellularly, whereas amastigote-like forms are localized within immune cells (Klatt et al. 2019).

Among sand fly hosts, the life cycle of *L. tarentolae* is relatively well understood. The development of *L. tarentolae* in three species of *Phlebotomus* has been described, providing valuable insights into parasite–vector interactions (Ticha et al. 2021).

Sauroleishmania species have traditionally been classified as hypopylarian, with their development confined to the hindgut of the sand fly vector (Wellcome Collection, n.d.). Nevertheless, Ticha *et al.* (2021) investigated the development of *Leishmania tarentolae* in three *Phlebotomus* species (*P. papatasi*, *P. sergenti*, and *P. perniciosus*) as well as in *Sergentomyia minuta* sand flies. Their study demonstrated that the development of *L. tarentolae* in *Phlebotomus* species can be characterized as peripylarian, with the parasite occurring in both the anterior and posterior midgut, as well as at the stomodeal valve (Ticha et al. 2021). Similar results were observed in female *S. minuta* sand flies, where anterior migration of the parasite was detected.

During experimental infections of sand flies, five distinct morphological forms of *L. tarentolae* were identified. Seven days post-infection, both long and short nectomonad forms were

present, considered the infective forms for the vertebrate host. However, metacyclogenesis has never been documented in *Sauroleishmania*, and it remains unclear whether these forms are indeed infectious to reptiles(Ticha et al. 2021).

4.2 Biotechnological applications of *Leishmania tarentolae*

Recently, *L. tarentolae* has been increasingly used as a model organism for investigating *Leishmania* biology and for the development of biotechnological applications. Its non-pathogenic nature allows cell cultivation under biosafety level 1 (BLS1) conditions, making it a competitive alternative to mammalian cell lines (Klatt et al. 2019). Moreover, its post-translational modifications (PTMs) closely resemble those found in mammals, particularly regarding glycosylation and protein folding processes. *L. tarentolae* exhibits also a high growth rate and requires minimal maintenance, resulting in reduced operational costs. These characteristics enable efficient scale-up processes, facilitating scale-up and production through cultivation in bioreactor systems (Klatt et al. 2019).

4.2.1 RNA editing

This process involves the post-transcriptional insertion and deletion of uridine residues in mitochondrial mRNAs. RNA editing is directed by small guide RNAs (gRNAs) that are encoded by the kinetoplast's maxicircle and minicircle DNA components. The reaction is catalyzed by a large multiprotein complex known as the editosome, which includes endonucleases, exonucleases, terminal uridylyl transferases, and RNA ligases. This editing mechanism is essential for generating translatable mRNAs from cryptic mitochondrial transcripts and plays a central role in the expression of mitochondrial genes. Owing to the abundance of kinetoplast DNA and the ease of culturing *L. tarentolae*, this species is widely used as a model organism to study the molecular mechanisms and evolutionary significance of RNA editing in trypanosomatids as studied by Simpson et al. (2004).

4.2.2 Gene amplification

In the kinetoplast, minicircles are present in very high copy numbers, making them an attractive system for the amplification of genes inserted into this region. Several biotechnological approaches exploit plasmid vectors designed to integrate into minicircles. Once integrated, the

gene is maintained and replicates together with the minicircle DNA, resulting in a natural and consistent amplification of the gene of interest. This leads to high levels of protein expression, which is valuable for antigen production, recombinant protein synthesis, and various experimental applications.

At the same time, the highly organized and genetically stable structure of thew kinetoplast, allows gene amplification to be maintained without the need for strong selective pressure, in contrast to many other eukaryotic systems. Consequently, gene amplification has been exploited for several purposes, including high-yield antigen production, large-scale recombinant protein expression, the generation of cell lines with strong overexpression of exogenous genes, and functional studies requiring multiple copies of a transgene (Singh et al. 2016).

4.2.3 Recombinant protein production

A wide range of expression systems for recombinant proteins has been developed for heterologous genes in both prokaryotic and eukaryotic hosts, including bacteria, yeast, mammalian cells, insect cells, and plant cells (Basile and Peticca 2009b). Compared with conventional expression systems, *L. tarentolae* offers several advantageous features, including low maintenance costs, a rapid growth rate in aerated conditions at 26 °C in suspension cultures, and suitability for scalable production with high recombinant protein yields. In addition, its glycosylation pattern closely resembles that of pathogenic trypanosomatids while maintaining significant similarity to mammalian glycosylation, making it particularly suitable for the expression of eukaryotic proteins requiring post-translational modifications (Mendoza-Roldan et al. 2022).

In this context, Jena Bioscience introduced, for the first time, a commercial kit for recombinant protein expression based on *Leishmania tarentolae*, promoted under the name LEXSY. This system enables both constitutive and inducible protein expression, as well as extracellular and secretory production. It employs the P10 strain, which is derived from the TARII/UC strain originally isolated by Parrot from the gecko *Tarentola mauritanica* (Klatt et al. 2019*)*. Efficient genetic transformation and heterologous protein expression were achieved by exploiting the regulation of gene expression by RNA polymerase I, a hallmark of *Leishmania* species and related kinetoplastid protozoa. In fact, through a double-crossover homologous recombination event, the expression cassette can be integrated into specific genomic loci of *L. tarentolae*.

Depending on the expression system employed, integration occurs either within the chromosomal 18S ribosomal RNA locus (*ssu*; constitutive expression system) or within the ornithine decarboxylase locus (*odc*; inducible-integrative system). The *ssu* locus is a repetitive genomic region characterized by high transcriptional activity driven by the host RNA polymerase I, whereas the *odc* locus is under the control of the RNA polymerase II. Moreover, transcription in *Leishmania* is polycistronic: the genome lacks introns, and consequently, cis-splicing does not occur. Instead, the processing of pre-mRNA involves trans-splicing and polyadenylation events that take place within intergenic regions. The structural organization of these intergenic sequences can further modulate the efficiency of protein expression (Klatt et al. 2019).

An alternative expression vector based on an inducible system was also developed. In this system, protein expression is activated by the addition of tetracycline, similar to the *E. coli* T7 RNA polymerase/tetracycline-controlled transcriptional activation (TET) repressor system (Klatt et al. 2019).

Examples of proteins successfully expressed using the LEXSY system include viral antigens, such as the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein, which has been employed for antibody production and studies of molecular interactions (Varotto-Boccazzi et al. 2021); viral capsid proteins that assemble into virus-like particles (VLPs) serving as vaccine candidates (Baechlein et al. 2013); antigens from *Trypanosoma cruzi*, used in serological assays for Chagas disease (Rooney et al. 2015); and antigens from *Leishmania* species, applied in immunological research and vaccine development (Rezaei et al. 2019). These recombinant proteins retain structural and functional properties closely resembling their native counterparts, owing to the eukaryotic folding and post-translational modification machinery provided by *L. tarentolae*.

4.2.4 Development of vaccine candidates

4.2.4.1 Vaccine candidates against human and canine leishmaniasis

Leishmania tarentolae has been recognized as a potential candidate for vaccine development against both human and canine *Leishmania*ses. Cross-protective immunity among *Leishmania* species is well documented, at both the molecular level and through the use of parasite-derived antigens. For example, the LBSap vaccine, which incorporates antigens from *L. braziliensis*, has demonstrated protective efficacy against *L. infantum*, highlighting the

feasibility of heterologous immunity (Nico et al. 2014; Aguilar-Be et al. 2005). Genomic analyses further indicate that *L. tarentolae* shares approximately 90% of its genes with pathogenic species such as *L. infantum* and *L. major*, while lacking several genes linked to virulence, supporting its favorable safety profile (Raymond et al. 2010). Moreover, even if considered non-pathogenic in mammals, *L. tarentolae* can infect macrophages and dendritic cells, completing the amastigote stage within these host cells (Breton et al. 2005).

The potential use of *L. tarentolae* as a surrogate pathogen has been supported by several preclinical investigations. Breton et al. (2005) showed that live promastigotes are efficiently internalized by dendritic cells in vitro, promoting cell maturation, including upregulation of MHC class II and co-stimulatory molecules. The same study further showed that intraperitoneal inoculation of *L. tarentolae* in BALB/c mice induce a polarization of the immune response toward Th1 pathway and provided significant protection against *L. donovani* (Breton et al. 2005). Subsequently, other studies demonstrate that live *L. tarentolae* - either alone or with adjuvants - can elicit cross-protective immunity against *L. major* (Haghdoust et al., 2022; Keshavarzian et al., 2020). Engineered *L. tarentolae* strains expressing antigens from human-pathogenic species or immunomodulatory molecules such as sand fly salivary proteins, have also been tested beyond wild-type strains (Salari et al. 2020; Saljoughian et al. 2013; Montakhab-Yeganeh et al. 2017; Katebi et al. 2015). In animal models, these engineered strains have been shown to confer protective immunity against pathogenic species, including *L. infantum* and *L. major*.

4.2.4.2 Anti-viral vaccines

L. tarentolae has also been investigated as a potential platform for the development of anti-viral vaccines, including those targeting human immunodeficiency virus type 1, human papillomavirus, hepatitis C virus, and coronaviruses (Breton et al. 2007; Bolhassani et al. 2011; Ansari et al. 2019; Epis et al. 2022). In these studies, engineered *L. tarentolae* was employed either as a delivery vehicle for the produced protein antigen or as a biofactory for antigen production. In the former approach, *L. tarentolae* targets dendritic cells, facilitating the delivery of specific antigens to secondary lymphoid organs. In the latter strategy, *L. tarentolae* functions exclusively as a production platform: the antigen of interest is expressed, subsequently purified, and then used in its isolated form.

Overall, these studies produce encouraging results in animal models, with the induction of virus-neutralizing antibodies.

4.2.5 Screening for anti-*Leishmania* drugs

Leishmania tarentolae has emerged as a valuable model organism for high-throughput screening of potential anti-*Leishmania*l compounds. Its genetic and metabolic pathways closely resemble those of pathogenic *Leishmania* species, while posing minimal biosafety concerns, making it particularly suitable for in vitro pharmacological studies. Moreover, *L. tarentolae* can be cultured rapidly under axenic conditions, enabling scalable screening of small molecules, natural products, and candidate drugs (Taylor et al., 2010).

Several studies have shown that amastigotes can reliably predict drug efficacy, showing strong correlation with responses observed in pathogenic species. Both axenic and intracellular amastigotes have been evaluated as a screening model in contexts with biosafety level 2 (BSL-2) not guaranteed. The activity of pentavalent antimony and amphotericin B was tested against *L. tarentolae* amastigotes, alongside human-pathogenic species *L. panamensis* and *L. braziliensis*, revealing comparable drug susceptibilities. These findings suggest that this non-pathogenic species can serve as a practical surrogate for the initial screening of anti*Leishmania*l compounds (Taylor et al. 2010).

In addition, large-scale compound screening using *L. tarentolae* promastigotes has successfully identified numerous active molecules later validated against pathogenic *Leishmania* (St. George et al. 2006). Moreover, high-throughput screening platforms incorporating *L. tarentolae* have been developed to rapidly evaluate extensive compound libraries, offering advantages in cost, safety, and early-stage drug discovery (Siqueira-Neto et al. 2010). Together, these studies highlight *L. tarentolae* as a surrogate model enabling efficient identification and characterization of novel therapeutics against leishmaniasis.

5 Sand flies

Sand flies are small hematophagous insects belonging to the order Diptera, family Psychodidae, and subfamily Phlebotominae. They are of major medical and veterinary significance due to their role as vectors of *Leishmania* parasites and other pathogens.

Currently, about 1000 species of sand flies have been described worldwide, 530 of which occurs in the New Word (Shimabukuro et al. 2017).

The subfamily *Phlebotominae* is classified into six genera: *Phlebotomus* (13 subgenera), *Sergentomyia* (10 subgenera), and *Chinius* (four species) from the Old World, and *Lutzomyia* (26 subgenera and groups), *Brumptomyia* (24 species), and *Warileya* (6 species) from the New World (Cecílio et al. 2022). Among these, the genera *Lutzomyia*, *Phlebotomus*, and *Sergentomyia* include several anthropophagous species. This feature makes them particularly noteworthy, as they play a crucial role in the transmission of diseases to humans (Cecílio et al. 2022).

Adult sand flies measure approximately 3 mm in length and are characterized by hairy bodies and wings. They possess a humped thorax and long, slender legs. The wings are narrow, lanceolate, and held at an angle above the body when at rest, forming a characteristic “V” shape (Cecílio et al. 2022).

Both males and females feed on plant nectar. However, females also require blood meals from mammals, reptiles, amphibians or birds to develop their eggs. Unlike males, female sand flies are hematophagous and possess piercing-sucking mouthparts adapted for blood feeding.

Nocturnal by nature, sand flies are typically found in dark and humid environments such as animal burrows, tree holes, and beneath rocks. Larval development requires a moist habitat rich in decomposing organic matter (Cecílio et al. 2022).

The life cycle of sand flies can be divided into four main stages: egg, larval, pupal, and adult. Approximately 5-8 days after a blood meal, female lay 30-70 eggs in protected, humid sites. The eggs hatch 4–20 days after being laid, giving rise to larvae that undergo four developmental instars. Depending on the species, larval development is completed within 20–30 days, after which the larva transforms into a pupa, a non-feeding, resting stage where metamorphosis occurs. Adults typically emerge during the hours of darkness, just before dawn. Males survive for about one week, whereas females live longer, as they can undergo more than one gonotrophic cycle, sometimes up to three (Cecílio et al. 2022).

6 Rational and aim of the thesis

Protozoan parasites of the genus *Leishmania* include several species of medical and veterinary relevance. In this context, further investigations are essential to expand the current knowledge about these parasites.

Leishmania tarentolae, a non-pathogenic species and primarily associated with reptiles, has emeged as a valuable model due to its ability to retain biological and molecular characteristics of pathogenic species while offering many advantages such as an easy manipulation (biosafety level 1) and a rapid growth rate. Despite this, important knowledge gaps remain regarding its biology and potential applications. Addressing these gaps is crucial for exploiting *L. tarentolae* as a platform for recombinant protein production, immunomodulation, live vaccination, and advanced molecular diagnostics.

Therefore, this thesis aims to investigate the biotechnological and biomedical potential of *Leishmania tarentolae*, together with the study of its epidemiology, providing evidence to support its use as a safe model system.

To address these abjectives, the thesis is structured in three main topics:

6.1 *L. tarentolae* biotechnological tool

This section examines *L. tarentolae* as a cell factory for protein production and as a platform for innovative vaccine delivery systems.

Studies include the development of chemically defined media to enhance growth and protein yield, as well as on the use of *L. tarentolae* as a vector for mucosal delivery of antigens, exemplified by the experimental LeCoVax-2 vaccine against SARS-CoV-2.

Scientific articles published in this section are:

1. **A novel chemically defined medium for the biotechnological and biomedical exploitation of the cell factory *Leishmania tarentolae*.**

   Cattaneo Giulia Maria, Varotto-Boccazzi Ilaria, Molteni Riccardo, Ronchetti Federico, Gabrieli Paolo, Mendoza-Roldan Jairo Alfonso, Otranto Domenico, Montomoli Emauele, Bandi Claudio, Epis Sara.

   [Published in: Scientific Report]

2. **Efficacyof mucosal vaccination using a protozoan parasite as a vehicle for antigen delivery: IgG and neutralizing response after rectal administration of LeCoVax-2, a candidate vaccine against COVID-19.**

   Epis Sara, Varotto-Boccazzi Ilaria, Manenti Alessandro, Rubolini Diego, Gabrieli Paolo, **Cattaneo Giulia Maria**, Gourlay Louise, Dapporto Francesca, Monti Martina, RazzanoIlaria, Leonardi Margherita, Iannacone Matteo, Recordati Camilla, Bertola Luca, Fiorina Paolo, Marvasi Luigi, Montomoli Emanuele, Zuccotti Gianvincenzo, Bandi Claudio.

   [published in: Pharmacological Research]

6.2 *L. tarentolae* biomedical applications

This section investigates the interactions of *L. tarentolae* with host immune cells and its potential as a mucosal vaccine vector.

Studies presented in this section investigate the Th1-mediated immune response induced by rectal administration of *L. tarentolae* and explore *L. tarentolae* intracellular persistence in canine macrophages, providing insights into host–parasite interactions and vaccine development strategies.

Scientific articles included in this section are:

1. **Rectal Administration of *Leishmania* Cells Elicits a Specific, Th1-Associated IgG2a Response in Mice: New Perspectives for Mucosal Vaccination against** leishmaniasis**, after the Repurposing of a Study on an Anti-Viral Vaccine Candidate.**

   Authors: Varotto-Boccazzi Ilaria, Epis Sara, **Cattaneo Giulia Maria**, Guerrini Noemi, Manenti Alessandro, Rubolini Diego, Gabrieli Paolo, Otranto Domenico, Zuccotti Gianvincenzo, Montomoli Emanuele, Bandi Claudio.

   [Published in: *Tropical Medicine and Infectious Disease*]

2. **Intracellular persistence of *Leishmania tarentolae* in primary canine macrophage cells.**

   Louzada-Flores Viviane Noll, Latrofa Maria Stefania, Lucente Maria Stella, Dambrós Bibiana Paula, Mendoza-Roldan Jairo Alfonso, Varotto-Boccazzi Ilaria, **Cattaneo Giulia Maria**, Späth Gerald F, Buonavoglia Alessio, Otranto Domenico.

   [published in: Acta Tropica]

6.3 Epidemiological and diagnostic applications

This part studies on the development of sensitive molecular detection methods, such as ddPCR for simultaneous identification of *L. infantum* and *L. tarentolae*, and presents field studies investigating sand fly fauna and the prevalence of *Leishmania* spp. in Northern Italy, illustrating the role of *L. tarentolae* in surveillance and epidemiological studies. In addition, considering the potential expansion of its host range, studies were carried out on non-traditional *Sauroleishmania* hosts, including fire salamanders and African geckos, to investigate their infection status and ecological distribution. Part of these epidemiological investigations were carried out during a three months research period in South Africa at the University of Free State (Bloemfontein), where field and laboratory studies were carried out on *Sauroleishmania* spp. in gecko hosts within a Southern African epidemiological context.
Published scientific articles present in this section are:

1. **Development of a novel ddPCR assay for simultaneous detection of the protozoan parasites *Leishmania infantum* and *Leishmania tarentolae*.**

   Alvaro Alessandro, **Cattaneo Giulia Maria**, Varotto-Boccazzi Ilaria, Molteni Riccardo, Mendoza-Roldan Jairo Alfonso, Brilli Matteo, Giovagnoli Virginia, Manenti Alessandro, Otranto Domenico, Bandi Claudio, Epis Sara. [Published in: Parasite and Vectors]

2. **Beyond reptiles: the fire salamander as a potential host for *Leishmania* (*Sauroleishmania*) *tarentolae*.**

   Alessandro Alvaro, **Giulia Maria Cattaneo**, Fabio Bigoni, Riccardo Molteni, Matilde Silvia Conconi, Domenico Otranto, Jairo Alfonso Mendoza-Roldan, Gentile Francesco Ficetola, Paolo Gabrieli, Claudio Bandi, Raoul Manenti, Sara Epis. [published in: International Journal for Parasitology: Parasites and Wildlife]

3. **Beyond reptiles: the fire salamander as a potential host for *Leishmania* (*Sauroleishmania*) *tarentolae***

   Alessandro Alvaro, **Giulia Maria Cattaneo**, Fabio Bigoni, Riccardo Molteni, Matilde Silvia Conconi, Domenico Otranto, Jairo Alfonso Mendoza-Roldan, Gentile Francesco Ficetola, Paolo Gabrieli, Claudio Bandi, Raoul Manenti, Sara Epis. [published in International Journal of Parasitology: Parasites and Wildlife]

4. **Unveiling *Sauroleishmania* in southern Africa: An investigation and supplementary description of *Leishmania* (*Sauroleishmania*) zuckermani from the gecko host *Chondrodactylus bibronii***

Bernard J. Jordaan, Alessandro Alvaro, **Giulia Cattaneo**, Monique Barnard, Sara Epis, Edward C. Netherlands. [published in International Journal of Parasitology: Parasites and Wildlife]

This eight articles are attached in the present thesis in the section “Publications”.

During the PhD, additional projects in parasitology and immunology led to three further publications, included in the section ‘Other Publications of Parasitological and Immunological Interest.

7 Publications

7.1 *Leishmania tarentolae* as a Biotechnological Platform and a Model for Diagnostic and Epidemiological Applications

7.1.1 A novel chemically defined medium for the biotechnological and biomedical exploitation of the cell factory *Leishmania tarentolae*.

Giulia Maria Cattaneo[1], Ilaria Varotto-Boccazzi[1,2], Riccardo Molteni[1], Federico Ronchetti[1], Paolo Gabrieli[1,2], Jairo Alfonso Mendoza-Roldan[3], Domenico Otranto[3,4], Emanuele Montomoli5,6, Claudio Bandi1,2 & Sara Epis1,2*

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Abstract

The development of media for cell culture is a major issue in the biopharmaceutical industry, for the production of therapeutics, immune-modulating molecules and protein antigens. Chemically defined media offer several advantages, as they are free of animal-derived components and guarantee high purity and a consistency in their composition. Microorganisms of the genus *Leishmania* represent a promising cellular platform for production of recombinant proteins, but their maintenance requires supplements of animal origin, such as hemin and fetal bovine serum. In the present study, three chemically defined media were assayed for culturing *Leishmania tarentolae*, using both a wildtype strain and a strain engineered to produce a viral antigen. Among the three media, Schneider's *Drosophila* Medium supplemented with Horseradish Peroxidase proved to be effective for the maintenance of *L. tarentolae* promastigotes, also allowing the heterologous protein production

by the engineered strain. Finally, the engineered strain was maintained in culture up to the 12th week without antibiotic, revealing its capability to produce the recombinant protein in the absence of selective pressure.

Keywords *Leishmania* culture, *Leishmania tarentolae*, Chemically defined medium, Protein expression, Promastigotes, Cell factory

Introduction

Leishmania tarentolae is a kinetoplastid protozoon belonging to the subgenus *Sauroleishmania*1. This species is associated with reptiles, especially lizards (Sauria), is not pathogenic to mammals, including humans, and is characterized by having a rapid growth rate in in vitro culture and low maintenance costs, resulting in an excellent candidate for biotechnological applications2,3. Commonly, *Leishmania* parasites are maintained in vitro as promastigotes, exploiting a liquid medium (e.g., RPMI 1640 medium, Schneider's insect medium, M199, DMEM and BHI) supplemented with antibiotics, sera of animal origin (e.g. fetal bovine serum—FBS, fetal calf serum—FCS), and, in some cases, other components such as l-glutamine, hemin and urine4–11. These components provide the required nutrients not included in a classic medium and lead to growth at high cell densities, with the possibility of a scaling-up. Besides supplying the required nutrients for *L. tarentolae* cell growth, the use of animal serum entails some disadvantages. For example, FBS is one of the most expensive supplements for cell media and it is easily contaminated if not properly managed. Regarding the ethical perspective, it should also be considered how FBS collection occurs in bovine fetuses and how its demand is unsustainable for a global supply12–14. Furthermore, it is highly recommended that the use of animal-derived compounds and fluids is minimized, and possibly avoided, in cell lines maintenance, in the area of biopharmaceutical applications12,13. The use of chemically-defined media is thus recommended in these contexts. Moreover, one of the main drawbacks associated with the use of animal-derived substances is the potential presence of viruses or contaminating microorganisms, that could then be incorporated into the final product13. Media that are not derived from animals have already been used in the maintenance of different *Leishmania* species, as alternatives to the BHI medium. These include RPMI-1640, Dulbecco's Modified Eagle Medium (DMEM), Luria–Bertani (LB) broth and Schneider's Drosophila Medium5,6,8,9,14–17. However, as these media

require the addition of components of animal origin (e.g., sera or hemin), finding alternative supplements to them has turned out to be difficult to achieve14. Indeed *Leishmania* spp., like other hemoflagellates, are obligate parasites, adapted to grow inside specific hosts, obtaining specific cofactors and nutrients, among which iron. For the in vitro growth of *Leishmania* spp. This micronutrient is usually added in the form of iron-containing porphyrins, like heme, or through the animal serum, such as FBS or FCS18,19. The addition of this type of supplementation is mandatory for in vitro *Leishmania* maintenance: its absence not only inhibits *Leishmania* cell multiplication, but also leads to cell death. A pioneer study by Gaughan and Krassner18 identified two iron porphyrins catalase and peroxidase as promising alternatives to hemin, in order to supplement in BHI medium18. A soy protein isolate (SPI) was also identified as a possible alternative to FBS, for the culturing of *Leishmania donovani* promastigotes in RPMI-164014. Our aim was to optimize an animal-free medium for the maintenance of *L. tarentolae* promastigotes. To this purpose we tested different media, with different types of supplementations, determining growth parameters at different conditions. Considering the potential applications of *L. tarentolae* in the biothechnological and biopharmaceutical areas, we also determined the production of a recombinant protein by an engineered strain of the parasite.

Results and discussion

Adaptation of *Leishmania* strains to RPMI-1640 Medium and Schneider's Drosophila Medium + soy protein isolate

The adaptation of *Leishmania* to different media was verified by evaluating cell growth and the morphology at the promastigote stage. Firstly, we tested the RPMI and Schneider media, adding the SPI, following the DA protocol. As positive control, cells were grown respectively in RPMI and Schneider media supplemented with FBS. Both the SPI-supplemented media did not allow to obtain an effective culture of Lt-P10: promastigote cells appeared rounded, differently from cells maintained in the commonly used media (RPMI + FBS; Schneider + FBS), where cells display a normal, elongated promastigote morphology (Supplementary File 1A). In addition, the number of cells that were counted in the SPI-supplemented media at day 4 was visibly reduced compared to that after the inoculum. In summary, results show that the first two media that were tested are not suitable for *L. tarentolae* maintenance, at least in the present experimental

setting. As previously reported, our strategy was to proceed with SA only after achieving an at least partial success with DA. Similarly, the adaptation of the engineered Lt-RBD was performed only with media that resulted suitable for the growth of the wild type strain Lt-P10. Consequently, we did not continue the experiments with RPMI + SPI or Schneider + SPI.

Schneider's Drosophila Medium + Horseradish Peroxidase: sequential adaptation strategy

Schneider + HRP was successfully assayed in DA for the maintenance of both Lt-P10 and Lt-RBD. Therefore, both strains were then subjected to SA, using BHI as the control medium. For a proper comparison, the same number of cells was inoculated in the flasks with the two media (BHI; Schneider + HRP) and the number of cells/ ml was monitored twice a week by optical microscopy. The experiment was repeated three times. In all replicas we observed a reduction in the number of promastigotes in Schneider + HRP once we reached a medium in which the serum was absent, with complete replacement by HRP (Table 1). In order to present the progress in cell adaptation, Table 1 shows the mean number of cells/ml for a single replica. Lt-P10 and Lt-RBD promastigotes in Schneider + HRP were morphologically similar to the respective controls in BHI, presenting the form of elongated nectomonads, short nectomonads or metacyclic promastigotes (Supplementary File 1B). After maintenance in Schneider + HRP, *Leishmania* cells displayed limited motility, in some cases limited to flagellar movement. In contrast, both Lt-P10 and Lt-RBD in BHI displayed active motility. Once moved to a 100% serum free medium (Schneider + HRP), the number of cells/ml decreased in both Lt-P10 and Lt-RBD (Table 1, passage 10–13). Considering the low number of cells in Schneider + HRP cultures, from passage 10 (100% SFM), all the available cells were inoculated into a new medium for the next passage. Here, the number of cells/ml in Lt-P10 cultured in Schneider + HRP, decreased from 2.10 × 107 cells/ml (passage 10, Table 1) to 9.5 × 104 cells/ml (passage 13, Table 1). Similarly, in Lt-RBD cultured in Schneider + HRP, the number of cells/ml reduced from 3.20 × 108 cells/ml (passage 10, Table 1) to 4 × 104 cells/ml (passage 13, Table 1). Lt-P10 and Lt-RBD were therefore unable to replicate and survive once moved in Schneider + HRP. The SA protocol has already been applied in several studies, in different types of microorganisms and cells, thanks to some advantages, such as progressive adaptation to the new medium and minimal stress for the cells20–23. In our SA protocol, each step in serum reduction requires 3 passages in the new medium. Therefore, to achieve an adaptation to a medium with 80% reduction of the

serum, approximately 20 days are required (Table 1). In addition, to obtain the culturing in a serum-free medium, which is anyway not satisfactory, 30 days are required. In conclusion, these results, in agreement with the microscopical observations, indicate that maintenance of *L. tarentolae* in a completely chemically defined medium such as Schneider + HRP is not only difficult (or impossible) to achieve, but also time consuming.

	BHI				Schneider + HRP (SA protocol)				
	Lt-P10		Lt-RBD		Lt-P10		Lt-RBD		
No passages	No cells/ml	Std. Dev.	No cells/ml	Std. Dev.	No cells/ml	Std. Dev.	No cells/ml	Std. Dev.	SA step
1	8.5×10^{7}	2.12×10^{7}	1.85×10^{8}	7.78×10^{7}	1.65×10^{8}	4.95×10^{7}	7.50×10^{7}	7.07×10^{6}	80% SSM 20% SFM
2	1.51×10^{8}	6.36×10^{6}	1.51×10^{8}	6.36×10^{6}	1.40×10^{7}	2.83×10^{6}	6.30×10^{7}	1.7×10^{7}	80% SSM 20% SFM
3	1.49×10^{8}	2.76×10^{7}	1.36×10^{8}	3.54×10^{6}	6.65×10^{7}	3.54×10^{6}	8.15×10^{7}	1.91×10^{7}	80% SSM 20% SFM
4	1.63×10^{8}	2.12×10^{6}	1.17×10^{8}	1.84×10^{7}	8.72×10^{7}	1×10^{8}	1.90×10^{8}	2.12×10^{6}	50% SSM 50% SFM
5	1.76×10^{8}	1.98×10^{7}	1.03×10^{8}	2.33×10^{7}	1.35×10^{7}	3.54×10^{6}	3.50×10^{7}	0	50% SSM 50% SFM
6	1.80×10^{8}	5.66×10^{7}	2.10×10^{8}	8.49×10^{7}	3.05×10^{7}	4.95×10^{6}	6×10^{5}	0	50% SSM 50% SFM
7	1.70×10^{8}	1.63×10^{7}	2.10×10^{8}	1.41×10^{7}	5.2×10^{7}	7.07×10^{6}	3.95×10^{7}	4.95×10^{6}	20% SSM 80% SFM
8	2.50×10^{8}	8.49×10^{7}	2.60×10^{8}	7.07×10^{7}	1.48×10^{8}	6.36×10^{6}	8.05×10^{7}	2.33×10^{7}	20% SSM 80% SFM
9	1.40×10^{8}	4.24×10^{7}	2.40×10^{8}	0	1.7×10^{8}	2.38×10^{6}	2.70×10^{8}	9.9×10^{7}	20% SSM 80% SFM
10	2×10^{8}	1.13×10^{8}	4.95×10^{8}	3.54×10^{7}	2.10×10^{7}	2.83×10^{6}	3.20×10^{8}	8.49×10^{6}	100% SFM
11	1.08×10^{8}	1.06×10^{7}	6.85×10^{7}	2.19×10^{7}	5×10^{4}	4.24×10^{4}	4×10^{4}	0	100% SFM
12	2.7×10^{8}	2.12×10^{8}	1.55×10^{8}	7.07×10^{6}	3.15×10^{4}	3.54×10^{4}	6.50×10^{4}	2.12×10^{4}	100% SFM
13	1.17×10^{8}	1.34×10^{7}	1.50×10^{8}	1.70×10^{7}	9.5×10^{4}	2.12×10^{4}	4×10^{4}	2.83×10^{4}	100% SFM

Table 1. Cell density mean (No cells/ml) and standard deviation (Std. Dev.) of Lt-P10 and Lt-RBD strains in Schneider's Drosophila Medium following the sequential adaptation protocol (SA), from a 100% serum supplemented medium (SSM, Schneider + 10% FBS) to a 100% serum free medium (SFM, Schneider + HRP). BHI medium was used as reference.

Schneider's Drosophila Medium + Horseradish Peroxidase: direct adaptation strategy

Lt-P10 and Lt-RBD strains were successfully maintained the HRP-supplemented media, displaying an unaltered morphology compared with the same strains maintained in the control medium (BHI) (see below). The adapted Lt-P10 and Lt-RBD strains were then stored at − 80 °C. Then, a frozen Lt-RBD stock was reactivated in FBSsupplemented medium, readapted to the Schneider + HRP following a DA protocol, and assayed in comparison with un-frozen Lt-P10 and Lt-RBD, always in Schneider + HRP (control: reactivated Lt-RBD in Schneider + FBS). During

the 5 days, cells in Schneider + HRP resulted to be elongated nectomonads, short nectomonads and metacyclic promastigotes, such as in the conventional media (Fig. 1). During the week, *Leishmania* cells displayed a similar motility in the chemically defined medium and in the control media, for all the three strains considered (Lt-P10, Lt-RBD and the reactivated Lt-RBD). At the beginning and at the end of the week, *Leishmania* cells resulted to be in the stationary phase of the curve while in the middle of the week, cells were in the logarithmic phase. Coherently with the above observation, at the beginning and at the end of the week cells resulted to be more active (moving cells or static cells with active flagellum) than in the middle of the week (static with active flagellum). Starting from a cell density of 3.2 × 106 cells/ml, the three strains resulted to replicate successfully in Schneider + HRP (0.40 mg/ml). The cell density, starting from 3.2 × 106 cells/ml, increased 8.3 times (2.67 × 107 cells/ml), 9.3 times (2.98 × 107 cells/ml) and 12.6 times (4.02 × 107 cells/ml), respectively for Lt-P10, Lt-RBD and the reactivated Lt-RBD resulted (Table 2). As shown in Fig. 2a, a significant difference in growth was observed between BHI and Schneider + HRP for both Lt-P10 and Lt-RBD at 24, 48, 72 and 96 h (for Lt-P10: $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively; for Lt-RBD: $p < 0.01$ for all time points except for 96 h with $p < 0.001$). In contrast, for reactivated Lt-RBD, no significant differences were observed between the serum supplemented medium and Schneider + HRP, at 24, 48 and 96 h; the only significant difference observed was at 72 h ($p < 0.05$) (Fig. 2a). In summary: (i) the reactivated Lt-RBD appears to display the best growth performance in the chemically-defined medium Schneider + HRP; (ii) the DA protocol guarantees optimal adaptation of *L. tarentolae* to Schneider + HRP (albeit with a lower growth efficiency compared to the classical BHI), and is also suited to obtain cells of the parasite suitable for long storage of *Leishmania* at − 80 °C. After 96 h, RBD protein expression in Lt-RBD and in the reactivated Lt-RBD grown in Schneider + HRP and in the conventional media (BHI and Schneider + FBS) was determined by Western blot analysis on cell pellets. For a proper comparison, the optical density (OD) from each culture was determined to compare the protein production from the same number of cells. The effective expression of RBD protein in all the conditions tested was confirmed: in the four samples tested, a unique band at 35 kDa, corresponding to the molecular weight of the heterologous protein RBD, was identified as the SARS-CoV-2 Spike RBD, using a specific antibody (Fig. 2b, Supplementary File 3). Then, the relative quantification of the protein was obtained with the Image Lab program (version 6.0.1). RBD protein band expressed by the reactivated Lt-RBD in Schneider + FBS was used as a

reference band. As specified in Supplementary File 2, for the reactivated Lt-RBD, the estimated protein quantity in the chemically defined medium resulted 1.2 times more than that of the same strain in the conventional medium. Moreover, band intensity in Lt-RBD in BHI and in Schneider + HRP resulted to be respectively 2.2- and 1-48 folds than the reference band. In summary, RBD protein had been well expressed by Lt-RBD strains, in both the conventional medium and in the peroxidase-supplemented medium, as well as after storage at − 80 °C in Schneider + HRP (Supplementary File 2).

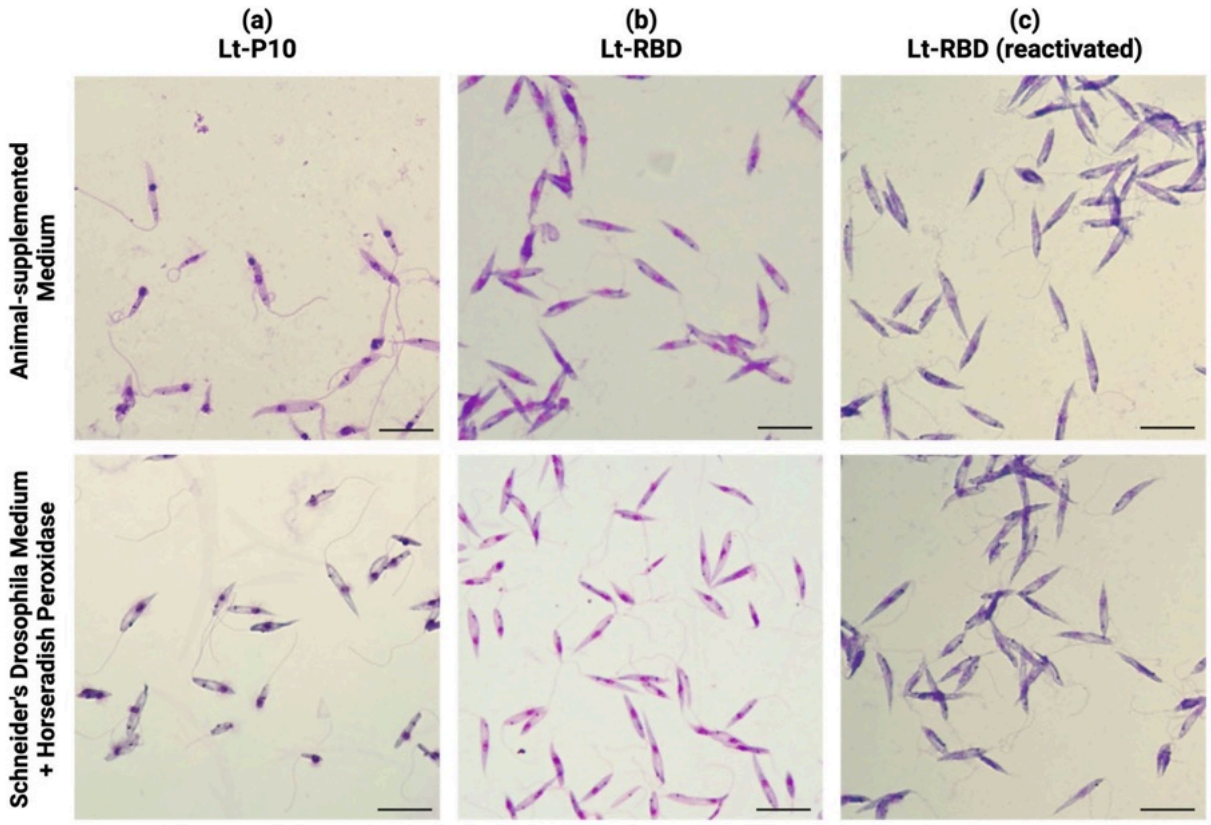

Figure 1. Morphological evaluation of Lt-P10, Lt-RBD and the reactivated Lt-RBD promastigotes 96 h after the inoculum in Schneider HRP, and in the media supplemented with animal components. Giemsa staining of: (a) Lt-P10 cultured in BHI and in Schneider + HRP (DA); (b) Lt-RBD promastigotes cultured in BHI medium and in Schneider + HRP (DA); (c) The reactivated Lt-RBD promastigotes cultured in Schneider + 10% FBS and in Schneider + HRP (DA); Scale bar: 10 µm.

Hours		0	24	48	72	96	Growth rate
Lt-P10 BHI	No cells/ml	3.2×10^{6}	1.51×10^{7}	1.09×10^{8}	1.8×10^{8}	2.02×10^{8}	63.13
	Std. Dev.	0	4.84×10^{6}	5.16×10^{7}	5.66×10^{7}	3.19×10^{7}	
Lt-P10 DA	No cells/ml	3.2×10^{6}	8.78×10^{6}	1.93×10^{7}	2.17×10^{7}	2.67×10^{7}	8.3
	Std. Dev.	0	1.76×10^{6}	6.92×10^{6}	1.50×10^{7}	1.28×10^{7}	
Lt-RBD BHI	No cells/ml	3.2×10^{6}	1.26×10^{7}	7.83×10^{7}	2×10^{8}	3.22×10^{8}	100.6
	Std. Dev.	0	5.25×10^{6}	3.74×10^{7}	8.81×10^{7}	1.20×10^{8}	
Lt-RBD DA	No cells/ml	3.2×10^{6}	5.33×10^{6}	1.92×10^{7}	1.42×10^{7}	2.98×10^{7}	9.3
	Std. Dev.	0	1.31×10^{6}	6.27×10^{6}	7.70×10^{6}	2.63×10^{7}	
React LT-RBD SCHN + FBS	No cells/ml	3.2×10^{6}	1.5×10^{7}	6.1×10^{7}	6.67×10^{7}	1.59×10^{8}	49.7
	Std. Dev.	0	9.59×10^{6}	3.16×10^{7}	2.25×10^{7}	1.01×10^{8}	
React Lt-RBD DA	No cells/ml	3.2×10^{6}	7.75×10^{6}	3.03×10^{7}	2.7×10^{7}	4.02×10^{7}	12.6
	Std. Dev.	0	2.06×10^{6}	2.54×10^{7}	1.66×10^{7}	2.05×10^{7}	

Table 2. Cell density mean (N° cells/ml), standard deviation (Std. Dev.) and growth rate of Lt-P10, Lt-RBD and the reactivated Lt-RBD strains during a week in Schneider's + HRP, during direct adaptation and in the conventional media (BHI and Schneider + FBS).

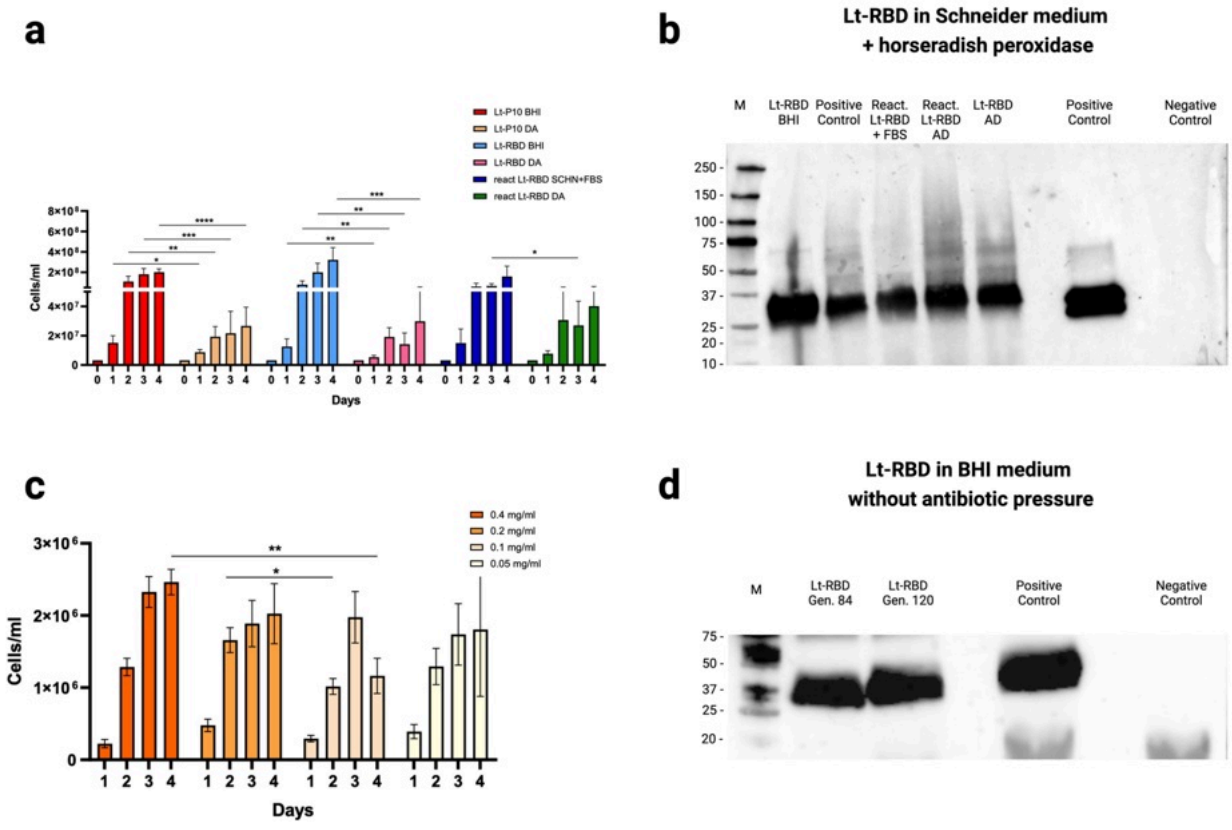

Figure 2. Comparison of cell growth in different media and evaluation of protein expression. (a) Growth curves of Lt-P10, Lt-RBD and reactivated Lt-RBD cultured in control media and in

chemically-defined medium (Schneider + HRP) exploiting a direct adaptation strategy, for a 5-day period starting from a concentration of 3.2 × 106 cell/ml. Bars show the mean ± SD. The significant differences were determined by t-test analysis. Differences were considered statistically significant when $p < 0.05$. The experiment was repeated three times. (b) RBD protein expression in Lt-RBD cells grown in Schneider + HRP (Direct Adaptation protocol) and in control medium (BHI and Schneider + FBS). (c) Growth curves of Lt-P10 cultured in Schneider at different Horseradish Peroxidase concentrations (0.4 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml) for a 5-day period starting from a concentration of 3 × 105 cells/ml. Bars show the mean ± SEM. The significant differences were determined by two-way ANOVA (Tukey's multiple comparisons test). Differences were considered statistically significant when $p < 0.05$. The experiment was repeated three times. (d) RBD protein expression in Lt-RBD grown in BHI without the addition of the antibiotic nourseothricin up to 120 cell generations.

Horseradish Peroxidase optimization

Different concentrations of HRP (0.40 mg/ml, 0.20 mg/ml, 0.10 mg/ml and 0.05 mg/ml) added to Schneider were tested on Lt-P10 culture monitoring cell survival and growth. At a concentration of 0.40 mg/ml and 0.20 mg/ ml cells grew respectively 12.31- and 10.13-folds from the beginning of the week, resulting active elongated nectomonad, short nectomonad and metacyclic promastigotes (Table 3). On the contrary, cells grown with 0.1 mg/ml and 0.05 mg/ml concentration of HRP, resulted to grow less than the other two conditions (growth rate at 5.8 and 9.03 respectively) (Table 3). At the two lowest concentrations (i.e. 0.10 mg/ml and 0.05 mg/ml), cells were metacyclic nectomonads, not active or only with an active flagellum. As described in Fig. 2c, cell growth was significantly greater at the condition of 0.40 mg/ml compared to 0.10 mg/ml on day 4 ($p < 0.05$). Similarly, a statistically significant difference in Lt-P10 growth is observed between 0.10 and 0.20 mg/ml on day 2 ($p < 0.01$) (Fig. 2c). Therefore, the addition of HRP at a final concentration of 0.40 mg/ml and 0.20 mg/ml to the chemically defined medium is recommended for cell adaptation without interfering with cell viability and morphology.

Day	0.40 mg/ml		0.20 mg/ml		0.10 mg/ml		0.05 mg/ml	
	No cells/ml	Std. Dev.	No cells/ml	Std. Dev.	No cells/ml	Std. Dev.	No cells/ml	Std. Dev.
0	2×10^5	0	2×10^5	0	2×10^5	0	2×10^5	0
1	2.24×10^5	1.66×10^5	4.78×10^5	2.45×10^5	2.94×10^5	1.24×10^5	3.91×10^5	2.76×10^5
2	1.29×10^6	3.40×10^5	1.66×10^6	4.89×10^5	1.02×10^6	3.13×10^5	1.29×10^6	7.12×10^5
3	2.33×10^6	6.02×10^5	1.89×10^6	9.11×10^5	1.98×10^6	1.01×10^6	1.74×10^6	1.20×10^6
4	2.46×10^6	4.98×10^5	2.03×10^6	1.17×10^6	1.16×10^6	6.89×10^5	1.81×10^6	2.62×10^6
Growth rate	12.31		10.13		5.81		9.03	

Table 3. Mean cell density, standard deviation (Std. Dev.) and growth rate of Lt-P10 cells cultured in Schneider's Drosophila Medium at different Horseradish Peroxidase concentrations.

Antibiotic removal

The technology used for the engineering of Lt-RBD strain exploits the chromosomal integration of a target gene in association with an antibiotic resistance gene. The use of the antibiotic nourseothricin allows thus the target gene maintenance and consequently the RBD protein expression24,25. However, for future medical and biotechnological applications of L. *tarentolae*, which implies its growth in industrial bioreactors, it is advisable the antibiotic resistance gene is removed26–28. As proved by other studies, engineered *Leishmania* appear to properly express recombinant proteins also in the absence of a selective antibiotic, in particular in case of a chromosomal integration of the target gene10,29. Therefore, Western blot analysis was carried out to verify the RBD protein production of Lt-RBD culture maintained without the use of nourseothricin for 12 weeks (corresponding to 120 generations of the parasite). As reported in Fig. 2d, RBD protein was secreted after 120 generations without antibiotic pressure, suggesting that engineered clones of *Leishmania* can survive and secrete proteins also in these growth conditions. Other studies proposed the generation of auxotrophic strains of *Leishmania*, by knocking out an essential metabolic gene, to be complemented through the incorporation of the required gene function in the selective cassette (Fig. 2d and Supplementary File 4)30. Overall, the search for a chemically defined medium for *Leishmania* maintenance resulted to be quite complex due to the necessary supplementation of specific nutrients such as iron. Three chemically defined media were tested as possible substitutes to BHI, with Schneider + HRP (0.40 mg/ml and 0.20 mg/ml) resulting to be the best alternative. In fact, this medium enables a good *L. tarentolae* replication without the use of animal components, also allowing the long storage and maintenance of *Leishmania* at − 80 °C. This

chemically-defined medium can also be used to maintain engineered strains, such as Lt-RBD, guaranteeing the production of recombinant proteins. As regards the engineered Lt-RBD, the heterologous protein production in absence of the selecting antibiotic was also verified, suggesting the possible removal of the antibiotic resistant gene in case of biotechnological applications of the cells at an industrial level. In conclusion, the efforts to set up optimal medium and culture conditions for *L. tarentolae* cultivation will improve the usage of this protozoon for large scale protein expression, which is a prerequisite for translational application in antigens, vaccines or therapeutics production.

Materials and methods

Leishmania tarentolae strains

Promastigotes of *L. tarentolae* strain P10 (Lt-P10; Jena Bioscience, Jena, Germany) isolated from Tarentola mauritanica (Moorish gecko) and the engineered strain *L. tarentolae* constitutively expressing the SARS-CoV-2 receptor-binding domain (Lt-RBD) were used in this study24,25. Both strains were maintained under aerated conditions, in 25 cm2 cell culture flask at 26 °C in Brain Heart Infusion Broth Medium (BHI) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with porcine hemin 5 µg/mL (Jena Bioscience, Jena, Germany) and 1% Penicillin–Streptomycin Solution (Euroclone, Milan, Italy). Lt-RBD culture was also supplemented with nourseothricin 100 µg/ml (Jena Bioscience, Jena, Germany) to select only the engineered clones expressing the heterologous protein RBD. The two strains were diluted in fresh BHI twice a week, monitoring their growth, motility and morphology using the optical microscopy.

Chemically-defined media tested

Three chemically-defined media were tested for the culture adaptation of *L. tarentolae* promastigotes: (i) RPMI1640 Medium + soy protein isolate; (ii) Schneider's Drosophila Medium + soy protein isolate; (iii) Schneider's Dros ophila Medium + Horseradish Peroxidase (see Fig. 3).

RPMI-1640 Medium + soy protein isolate

Leishmania tarentolae culture was tested in RPMI-1640 Medium (RPMI) (Euroclone, Milan, Italy) supplemented with 10% sterile Soy Protein Isolate (SPI) (My Protein, Manchester, UK), 1% l-

glutamine (Euroclone, Milan, Italy) and 1% Penicillin–Streptomycin solution (Euroclone, Milan, Italy). The same strain was also maintained in RPMI supplemented with 10% FBS (Euroclone, Milan, Italy), 1% l-glutamine (Euroclone, Milan, Italy) and 1% Penicillin–Streptomycin solution (Euroclone, Milan, Italy) and used as reference.

Schneider's Drosophila Medium + soy protein isolate

Schneider's Drosophila Medium (Schneider) (Thermo Fisher, Waltham, USA) was supplemented with 10% autoclaved SPI (My Protein, Manchester, UK), 1% Penicillin–Streptomycin solution (Euroclone, Milano, Italy) and tested for *L. tarentolae* maintenance. For comparison, Schneider supplemented with 10% FBS (Euroclone, Milan, Italy) and Penicillin–Streptomycin solution (Euroclone, Milan, Italy) was used.

Schneider's Drosophila Medium + Horseradish Peroxidase

Schneider (Thermo Fisher, Waltham, USA) added with Horseradish Peroxidase (HRP) (0.40 mg/ml; Thermo Fisher, Waltham, USA) was tested and the conventional BHI was used for comparison.

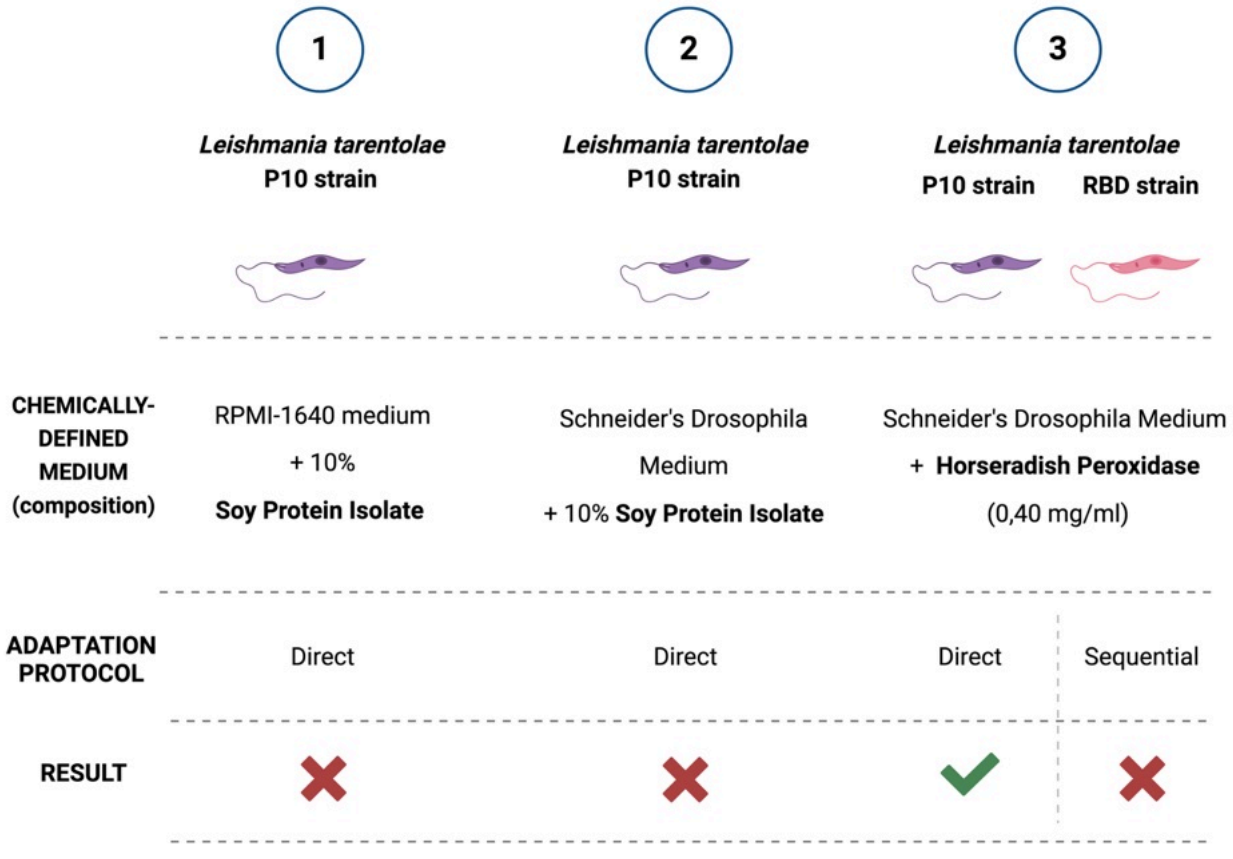

Figure 3. Summary of the chemically-defined media and the adaptation strategies (direct or sequential) tested on *Leishmania tarentolae*-P10 and on *Leishmania tarentolae*-RBD cultures.

Assays to adapt *Leishmania* strains to chemically-defined media

Two different strategies were tested for cell adaptation in the new media proposed: the direct adaptation strategy (DA) and the sequential adaptation strategy (SA) (see Fig. 3)31. In the DA protocol, *Leishmania* cells maintained in animal-supplemented medium were inoculated directly in the tested chemically-defined medium. The concentration of the cells, motility and morphology were determined and compared with that obtained with the reference medium (serum-supplemented medium). By contrast, in the SA protocol cells were adapted from a serum-supplemented medium to a serum-free medium through several steps, characterized by a decreasing percentage of animal serum added (80%, 50%, 20%, 0%). For proper comparison, cells were subcultured twice a week with the same number of cells. These chemically-defined media were tested on both Lt-P10 and the engineered Lt-RBD to verify their potential use also for the cultivation of strains engineered for heterologous protein expression. However, the SA protocol was adopted only when the DA adaptation was successful. Similarly, the adaptation of the engineered Lt-RBD was tested only in media successfully tried out on Lt-P10. The capability of the cells to grow in a chemically-defined medium was also evaluated after a long storage at − 80 °C. After a direct adaptation to Schneider + HRP, Lt-RBD was stored at—− 80 °C in this chemically defined medium following the protocol explained below. Briefly, 3.6 ml of cells (about 6 × 107 cells/ml) were added to 1.2 ml of sterile glycerol and aliquoted in three cryovials. Each cryovial was then stored at—− 80 °C using Corning CoolCell LX (Corning, New York, USA). The correct adaptation of stored Lt-RBD in Schneider + HRP was verified as follows: Lt-RBD stocks were kept in ice for 20 min and then inoculated in 10 ml of Schneider + 20% FBS in a 25 cm2 cell culture flask at 26 °C. After two passages in this medium, cells were re-adapted in Schneider + HRP exploiting the DA protocol.

Cell growth determination

Cell growth was monitored twice a week by optical microscopy observation, together with cell morphology and motility. Based on the morphology, cells were classified in three main categories: (1) elongated nectomonad; (2) short nectomonad and metacyclic promastigotes; (3) rounded metacyclic promastigotes and rounded promastigotes32. Then, *Leishmania*

motility was taken into consideration comparing moving cells, static cells, and static cells with active flagellum. Finally, once the adaptation was completed, a slide from each condition was prepared and stained with Giemsa (Carlo Erba, Milan, Italy), following standard protocols, to compare cell morphology.

Optimization of Horseradish Peroxidase concentration

After the adaptation of *Leishmania* to Schneider + HRP (0.40 mg/ml), the Lt-P10 strain was incubated in Schneider supplemented with four different concentrations of HRP to determine its optimal concentration: 0.40 mg/ml; 0.20 mg/ml; 0.10 mg/ml; 0.05 mg/ml. A 12-well plate was used for the experiment and 2 × 105 cells/ml were inoculated in each well with a final volume of 2 ml. As for the maintenance, the number of cells/ml, cell morphology and motility were monitored at the optical microscopy every day for a week.

Western Blot analysis

The RBD protein production was verified in Lt-RBD culture, compared to the Lt-P10 strain (non-engineered) by Western Blot analysis, as described in Varotto-Boccazzi and colleagues19,24. Briefly, cell culture was centrifuged at 3000g for 10 min, then cell pellet was resuspended in loading buffer (Thermo Fisher Scientific, Massachusetts, US) and boiled for 5 min. The samples were loaded to Mini-PROTEAN TGX Stain-Free Protein (Bio-Rad Laboratories, Hercules, California, US) and, after the electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California, US). After 5 min of incubation with EveryBlot Blocking Buffer (Bio-Rad Laboratories, Hercules, California, US), the membrane was incubated with SARS-CoV-2 Spike RBD antibody 1:5000 (Gene Tex, Irvine, California, US) (Bio-Rad Laboratories, Hercules, California, US) in blocking buffer for 2 h at room temperature. The membrane was then washed for three times with Phosphate Buffered Saline (PBS) supplemented with 0.1% of Tween 20 (PBS-T) and incubated for 1 h with Goat anti-Rabbit IgG Secondary antibody HRP 1:30,000 (Thermo Fisher Scientific, Massachusetts, US). After three washes in PBS-T, the membrane was incubated with Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, California, US) for 5 min and then analyzed with the ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Hercules, California, US). Subsequently, a quantitative analysis of RBD expression was carried out with Image Lab Software (version 6.0.1) (Bio-Rad Laboratories, Hercules, California, US).

RBD expression in the absence of the antibiotic selection.

The growth of the engineered Lt-RBD strain was verified in BHI without the addition of nourseothricin, the antibiotic for the selection of the engineered strain, for over 100 cell generations. Cells were thus monitored twice a week and the RBD expression was verified by Western Blot analyses, following the protocol described above, once a week for 12 weeks.

Statistical analysis

Statistical analyses were performed by t-test or by two-way ANOVA (followed by Tukey's multiple comparisons test) using GraphPad Prism 8.0 (GraphPad, CA, USA). A p value less than 0.05 was considered statistically significant for all the experiments.

Patent

The antigen Lt-RBD and its potential application have been described in the Patent N. IT 102021000004160.

Data availability

All data generated and analyzed during this study are included in the manuscript and its supplementary Information files.

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Author contributions

Conceptualization, S.E., I.V.B., G.M.C.; Methodology, I.V.B., G.M.C.; Formal analysis, P.G.; Investigation, R.M., F.R., G.M.C.; Resources, S.E., C.B; Writing—original draft preparation, G.M.C.; Writing—review and editing, S.E., I.V.B., C.B.; Supervision, E.M., J.M.R, D.O.; Funding acquisition, S.E., C.B.

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Competing interests

The author E.M. was employed by the company VisMederi Srl. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; nor in the decision to publish the results.

Additional information

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7.1.2 Efficacy of mucosal vaccination using a protozoan parasite as a vehicle for antigen delivery: IgG and neutralizing response after rectal administration of LeCoVax-2, a candidate vaccine against COVID-19

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Abstract

Mucosal vaccination is regarded as a promising alternative to classical, intramuscular vaccine delivery. However, only a limited number of vaccines have been licensed for mucosal

administration in humans. Here we propose *Leishmania tarentolae*, a protozoan parasite, as a potential antigen vehicle for mucosal vaccination, for administration via the rectal or oral routes. To test this hypothesis, we exploited *L. tarentolae* for the production and delivery of SARS-CoV-2 antigens. Two antigens were assayed in BALB/c mice: Lt-spike, a *L. tarentolae* clone engineered for the surface expression of the SARS-CoV-2 spike protein; RBD-SD1, a purified portion of the spike protein, produced by another engineered clone of the protozoon. Immune response parameters were then determined at different time points. Both antigens, administered either separately or in combination (Lt-spike + RBD-SD1, hereafter LeCoVax-2), determined significant IgG seroconversion and production of neutralizing antibodies after subcutaneous administration, but only in the presence of adjuvants. After rectal administration, the purified RBD-SD1 antigen did not induce any detectable immune response, in comparison with the intense response observed after administration of LeCoVax-2 or Lt-spike alone. In rectal administration, LeCoVax-2 was also effective when administered without adjuvant. Our results show that *L. tarentolae* is an efficient and safe scaffold for production and delivery of viral antigens, to be used as vaccines. In addition, rectal vaccination experiments prove that *L. tarentolae* is suitable as a vaccine vehicle and adjuvant for enteral vaccination. Finally, the combined preparation LeCoVax-2 can be considered as a promising candidate vaccine against SARS-CoV-2, worthy of further investigation.

Introduction

Mucosal epithelia represent both an important colonization site and the main access route to the human body for most infectious agents, from respiratory viruses to intestinal helminths [1,2]. For these types of infections, mucosal immunity is a first shield, that protects the body during the initial phases of pathogen colonization and spread [2–4]. Mucosal vaccine administration (e.g., through the oral or nasal routes) is regarded as an ideal means to stimulate an effective immune response at the mucosal level, whose major effectors against a vast array of pathogens are secretory IgA antibodies [5]. In addition, mucosal vaccination, particularly through the oral route, holds several potential practical advantages compared to classic intramuscular injection, which includes a simplification of vaccine production procedures (in terms of sterility and purity of the components), easier administration and the possibility of partially overcoming vaccination hesitancy. Despite the advantages of mucosal vaccination, less than ten mucosal vaccines have been licensed for human use, to date, and are limited to

intranasal influenza vaccines and to a few oral vaccines against intestinal pathogens [1]. This issue is also relevant to currently licensed COVID-19 vaccines, all designed for intramuscular administration [6–8]. These vaccines have indeed been very effective in reducing the incidence of severe disease and hospitalization [9,10] but have had less effects in the containment of asymptomatic or mild infections, and thus on viral circulation [3,11].

Considering the relevance of mucosal vaccination to confer protection against a vast array of infectious agents, we focused on an alternative vaccine platform, the protozoan parasite *Leishmania tarentolae*, which is possibly suitable for delivery through the enteral route. *L. tarentolae* is a reptile parasite and is not pathogenic to humans and other mammals [12,13]. This microorganism has already been manipulated for the expression of viral antigens and has been tested as a living vaccine vehicle in murine models, following classical subcutaneous injection (e.g., [14–17]). The rationale that led us to hypothesize that this parasite is suitable for enteral administration, as a vehicle for antigen delivery and vaccination, is as follows: (1) *Leishmania* cells, including the promastigote stage, are resistant to osmotic shock and chemical and mechanical insults, owing to the presence of interlinked microtubules beneath the plasma membrane [18] the robustness of these cells thus makes them suitable for administration through alternative routes, e.g. through the enteral route; (2) the size of *Leishmania* promastigotes lies in the range of the particles that are expected to be internalized by microfold (M) cells in the intestine, which are involved in the transfer of antigens from the gut lumen to mononuclear phagocytes in Peyer's patches [19]; (3) both classical and recent studies prove that infection by *Leishmania* parasites can be acquired by oral ingestion, in addition to the common and well-established transmission through the bite of sand flies [13,20–21]. The above issues, in particular the evidence of oral transmission in the natural cycle of L. *tarentolae*, strengthen the potential of this microorganism as a vehicle for the administration of antigens through an enteral route.

A further major feature that makes *L. tarentolae* a suitable vaccine vehicle is that, upon its inoculation into mammalian tissues, it is expected to target dendritic cells (DCs) and other phagocytic cells [14,22]. Indeed, the major niche for survival and replication of *Leishmania* species infecting mammals is represented by phagocytic myeloid cells, including DCs and macrophages [23,24]. This propensity to be phagocytized by mammalian DCs and macrophages has also been demonstrated for *L. tarentolae* [25,26]. In addition, commercial kits are available for the genetic transformation of L. *tarentolae*, for the production of

recombinant proteins [12,13]. Finally, protein glycosylation in *L. tarentolae* mimics that of mammals, which is an interesting feature for the production of viral antigens to be used as vaccines [12,13].

In a recent in vitro study, we showed that the Lt-spike clone of L. *tarentolae*, engineered for the surface expression of the spike antigen of SARS-CoV-2, effectively delivers the spike antigen into DCs [26]. This *L. tarentolae* clone thus represents a potential COVID-19 vaccine candidate, suitable for further testing in in vivo assays. Here, we present the results of a murine model study that assesses the capacity of Lt-spike to induce an effective and neutralizing antibody response, when administered either alone, or in combination with the receptor binding domain of the spike protein [27]. In addition to the classical, subcutaneous route of administration (which represents, in the mouse model, the surrogate for intramuscular delivery), we investigated the rectal route, with the aim of determining the potential of *L. tarentolae* as a vehicle for mucosal vaccination through the enteral route.

Materials and methods

Tested antigens and the LeCoVax-2 vaccine candidate

Three types of antigen preparations were assayed in this study: Ltspike; RBD-SD1 and LeCoVax-2 (Fig. 1A). Lt-spike is a clone of the *L. tarentolae* P10 strain, designed to express the whole spike protein of the SARS-CoV-2 virus at its surface. RBD-SD1 (hereafter RBD) is a purified polypeptide that includes both the receptor binding domain (RBD) and the SD1 portion of the spike protein. RBD was obtained by engineering the *L. tarentolae* P10 strain for secretory expression of RBD (LtRBD clone). In both cases, the source of the sequence to be cloned into *L. tarentolae* was the Whuan SARS-CoV-2 strain. LeCoVax-2 is composed by the two antigens described above, Lt-spike and RBD. For more details see [26,27]. Briefly, Lt-spike was obtained using the codon-optimized DNA sequence of the spike gene (GenBank: MN908947) with a GSAS substitution at the furin cleavage site (residues 682–685) and a C-terminal 6xHis-tag. The sequence was synthesized and cloned into the pLEXSY-sat2 vector (Jena Bioscience, Jena, Germany); engineered Lt-spike was grown in Brain Heart Infusion (BHI) liquid medium supplemented with 5 μg/ml porcine hemin, 50,000 U/l Penicillin, 50 mg/l Streptomycin and 100 μg/ml Nourseothricin (Jena Bioscience, Jena, Germany) at 26 ◦C in the dark under aerated conditions. Target protein expression was confirmed by Western blotting and immunofluorescence assays, as described in Varotto-Boccazzi et al. [26]. To obtain the Lt-RBD

clone, the gene coding for RBD-SD1 was codon-optimized, synthesized and subcloned into the pLEXSY-sat2 vector (Jena Bioscience, Jena, Germany) for constitutive, secreted expression. Cloning results in the incorporation of a C-terminal 6xHis-tag with a signal sequence from *Leishmania* mexicana, which allows the secretion of the target protein into the culture medium. To obtain the purified RBD-SD1 antigen, Lt-RBD were cultured (2.5 L) for 4 days at 26 ◦C in the dark, with constant agitation under aerated conditions. Purification of the secreted protein is reported in detail in Varotto-Boccazzi and colleagues [27]. Protein concentration was measured spectrophotometrically using a NanoDrop spectrophotometer, using the theoretically calculated A280 nm for 1 mg/ml Lt-RBD of 1.077 and with the BCA Protein Assay Kit (Merck). Finally, the *L. tarentolae* P10 (non-engineered strain; Lt-P10) was used as a control strain for all experiments. Both antigens (Lt-spike and RBD) were tested in BALB/c mice either separately, or as the combined preparation LeCoVax-2.

Mouse study designs

A preliminary experiment was performed to evaluate the immunogenicity and efficacy of the two Lt-spike and RBD antigens, administered alone, or in combination (LeCoVax-2), in the absence of adjuvant, testing two classical routes of administration: subcutaneous (SC) and intraperitoneal (IP). A second experiment was carried out testing the immunogenicity of LeCoVax-2 with adjuvants (AddaVax, Alum, R848; InvivoGen) with two routes of administration, rectal (R) or SC.

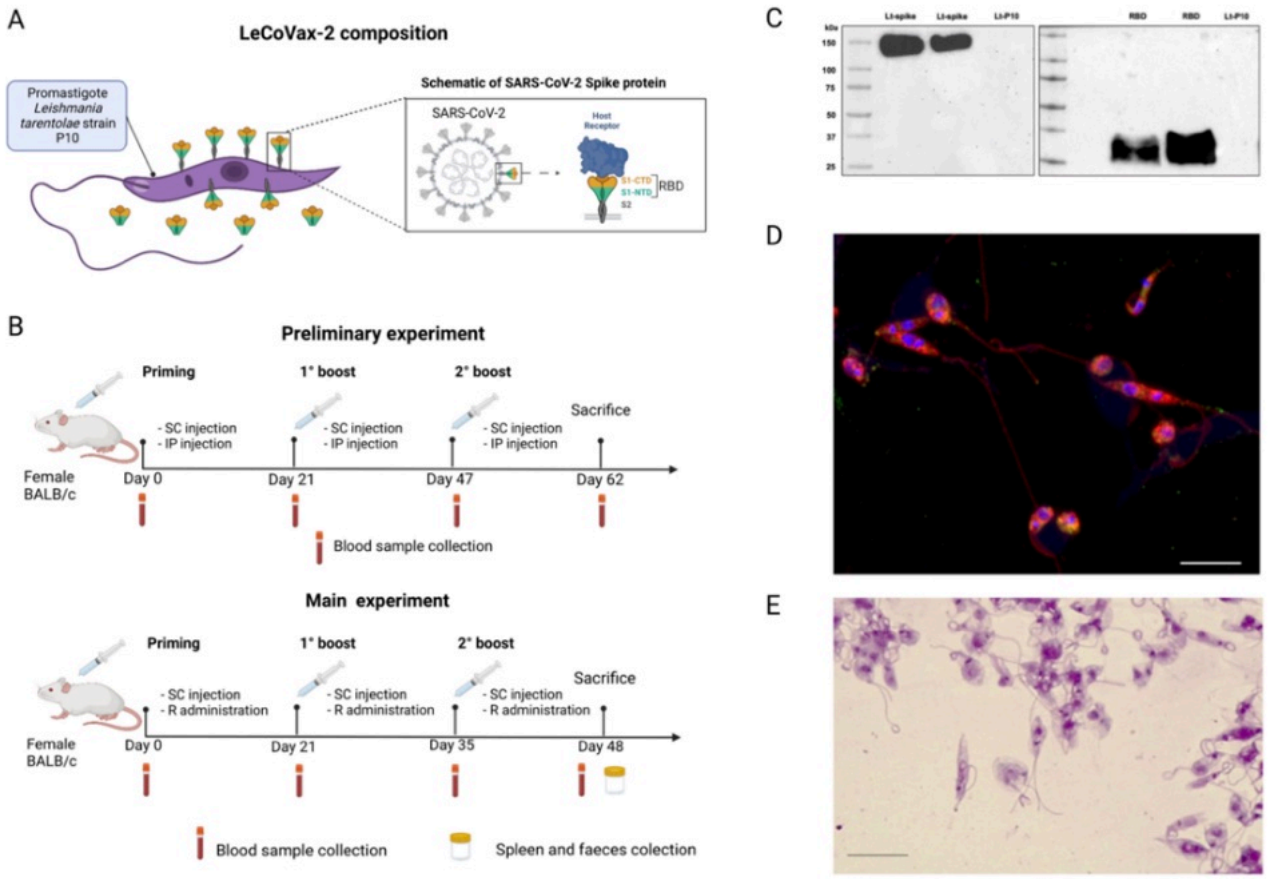

Fig. 1. Characterization of the LeCoVax-2 vaccine. (A) The LeCoVax-2 vaccine formulation is defined as the combination of the protozoan parasite *Leishmania tarentolae*, engineered to express the SARS-CoV-2 virus spike protein, and the purified recombinant protein RBD-SD1 overexpressed in L. *tarentolae*. (B) Schematic diagram of preliminary and main immunization experiments and sample collection, performed on BALB/c female mice. SC: subcutaneous injection; IP: intraperitoneal injection; R: rectal administration. (C) Expression of spike and RBD proteins were confirmed by Western blotting using a SARS Coronavirus spike protein polyclonal antibody. Lt-P10: *L. tarentolae* unengineered, control strain. (D) Evaluation of spike protein production by engineered *L. tarentolae* by immunofluorescence assay. Green dots show the presence of spike protein in the *Leishmania* cytoplasm, while orange/yellow signals represent the colocalization of spike protein to the membranes, which are labeled in red using Concanavalin A staining. Nuclear and kinetoplastid DNA were stained with DAPI (blue). (E) Giemsa staining of *L. tarentolae* engineered for the expression of spike protein of SARS-CoV-2 virus after treatment with formalin. The scale bar represents 10 µm.

Experimental group	Formulation	Dose/ volume
Subcutaneous injection		
PBS-SC	PBS solution (control)	100 µl
Lt-spike-SC	2×10^7 cells Lt-spike	100 µl
Lt-spike+RBD-SC	2×10^7 cells Lt-spike + 10 µg RBD (LeCoVax-2)	100 µl
RBD-SC	10 µg RBD	100 µl
Lt-P10-SC	2×10^7 cells Lt-P10	100 µl
Intraperitoneal injection		
Lt-spike-IP	5×10^6 cells Lt-spike	150 µl
Lt-spike-RBD-IP	5×10^6 cells Lt-spike + 10 µg RBD (LeCoVax-2)	150 µl
RBD-IP	10 µg RBD	150 µl

Lt: *Leishmania tarentolae*; RBD: Receptor Binding Domain

Table 1 Summary of the preliminary immunization experiment according to vaccine administration mode (n = 5 female mice per experimental group).

Mouse immunization

Animals and animal ethics statement. Female BALB/c mice (not germ-free), 8–10 weeks old, were housed at Farefarma's animal facility Emozoo (Casole d'Elsa, Siena). Each group of animals was housed in a cage in an animal facility (with limited access) on a 12-h light/dark cycle at room temperature (24 ± 2 ◦C) with constant humidity (40 ± 15%); animals were allowed free access to food and water. The animals were used according to Directive 2010/63/UE regarding the protection of animals used for experimental or other scientific purposes, enforced by the Italian Legislative Decree n◦ 26 of 2014 and Ministerial. Decree. Project authorizations: 859/2020-PR, phase B authorizations: D4A18. B.1YW; D4A18.B.FX6. At the end of the experiment the animals of all groups were anesthetized by isoflurane then sacrificed by cervical dislocation.

Preliminary immunization experiment. Animals were divided into eight groups (n = 5 mice per group) and received three primebooster immunizations on days 0, 21, 47, according to the modalities described in Table 1 and Fig. 1B. 62 days post-immunization, sera from the immunized mice were collected for subsequent analysis.

Main immunization experiment. Prior to immunization, *Leishmania* parasites were inactivated overnight with 0.15% formalin at 4 ◦C, then the cells were washed three times with PBS and

finally stored at 80 ◦C. To evaluate the integrity of the *Leishmania* cells after inactivation, a morphological evaluation was performed by Giemsa staining. Endotoxin determination was performed on recombinant RBD protein and Lt-spike with the Pierce Chromogenic Endotoxin Quant kit (ThermoFisher, USA). Animals were divided into ten groups (n = 10 mice per group) and were immunized by SC injection or rectal administration with different vaccine compositions, as reported in Table 2 and Fig. 1B. Briefly, five groups were subcutaneously immunized in the dorsal region of the mouse (three prime-boost immunization) on day 0, 21 and 35 with different treatments: i) Lt-spike and recombinant RBD antigen, mixed with AddaVax adjuvant (AddaVax; InvivoGen) at a 1:1 ratio (v:v); ii) Lt-spike and recombinant RBD antigen, mixed with aluminium phosphate gel adjuvant (Alum) (Adju-Phos 2%; InvivoGen) at a 1:1 ratio (v:v); iii) recombinant RBD antigen and AddaVax at a 1:1 ratio (v:v); iv) recombinant RBD antigen mixed with Alum at a 1:1 ratio (v:v); v) PBS as placebo. Five groups were rectally immunized on day 0, 21 and 35 with different formulation as follows: i) Lt-spike and recombinant RBD antigen mixed with 10 µg Resiquimod (R848, Invivogen); ii) Lt-spike with recombinant RBD antigen and 25 µg R848; iii) Lt-spike and recombinant RBD antigen without the addition of adjuvant; iv) recombinant RBD antigen mixed with 25 µg R848; v) PBS as placebo. Sera were collected on day 0 (prior to immunization), 21, 35 and 48 in 95 animals for characterization of the antibody response. The body weight of each mouse was measured on day 0 and every seven days. On the day of sacrifice (day 48), the spleens were collected and used to isolate splenocytes for peptide stimulation and subsequent ELISpot analysis, whereas the faeces were immediately frozen at 80 ◦C for IgA antibody response determination. Selected organs/tissues were sampled for histological examination.

In-house enzyme-linked immunosorbent assay (ELISA)

Serum IgG determination

An ELISA assay was used to determine the IgG antibody response in the sera of immunized mice. Briefly, 96-well ELISA plates were coated with 1 µg/ml recombinant SARS-CoV-2 Spike-RBD protein (Sino Biological) at 4 ◦C overnight. After three washes with Tris Buffered Saline (TBS) containing 0.05% (v/v) Tween 20 (T-TBS), coated plates were blocked for 1 h at 37 ◦C with a solution of T-TBS containing 5% (w/v) Non-Fat Dry Milk (NFDM, Euroclone). Murine serum samples were serially diluted from 1/50 up to 1/3200 in two-fold dilutions in T-TBS containing 5% NFDM. Plates were washed three times with T-TBS and 100 µl of each serial dilution were

added to the plates and incubated for 1 h at 37 ◦C. Plates were washed three times and 100 µl of goat antimouse IgG1 HRP-conjugated (Bethyl Laboratories) diluted 1:50,000 in 5% NFDM/T-TBS were added to each well and the plate was incubated at 26 ◦C for 30 min. Following incubation, plates were washed three times and 100 µl/well of 3,3',5,5' -Tetramethylbenzidine (TMB) substrate (Bethyl Laboratories) was added and incubated in the dark at room temperature (RT) for 20 min. The reaction was stopped by adding 100 µl of hydrochloric acid solution 0.5 M (Fisher Chemical) and read within 20 min at 450 nm with SpectraMax ELISA plate (Medical Device) reader. A cut-off value was obtained for each plate by multiplying by three the average of the blank optical density (OD) signal derived from wells not containing analyte (background) [28].

Experimental group	Formulation	Dose/ volume
Subcutaneous injection		
PBS-SC	PBS solution (control)	100 µl
LeCoVax-2 +Alum-SC	2×10^7 cells Lt-spike + 10 µg RBD + Alum	100 µl
LeCoVax-2 +AddaVax-SC	2×10^7 cells Lt-spike + 10 µg RBD + AddaVax	100 µl
RBD+Alum-SC	20 µg RBD + Alum	100 µl
RBD+AddaVax-SC	20 µg RBD + AddaVax	100 µl
Rectal administration		
PBS-R	PBS solution (control)	50 µl
LeCoVax-2-R	1×10^8 cells Lt-spike + 20 µg RBD	50 µl
LeCoVax-2 +R84810-R	1×10^8 cells Lt-spike + 20 µg RBD + 10 µg R848	50 µl
LeCoVax-2 +R84825-R	1×10^8 cells Lt-spike + 20 µg RBD + 25 µg R848	50 µl
RBD+R84825-R	20 µg RBD + 25 µg R848	50 µl

Lt: *Leishmania tarentolae*; RBD: Receptor Binding Domain; SC: subcutaneous; R: rectal; Alum: aluminium phosphate gel; AddaVax: squalene-based oil-in-water (w/o) nano-emulsion; R848: Resiquimod.

Table 2 Summary of the main immunization experiment (n = 10 mice per experimental group) according to vaccine administration mode.

IgA determination in faeces For the measurement of mucosal IgA, fresh fecal pellets were weighed and dissolved in a protease inhibitor solution (Roche), as reported in Tonti and colleagues [29]. After 10 min at RT, samples were vigorously vortexed; these two steps were repeated once more. Finally, samples were centrifuged at 13000 g for 5 min at 4 ◦C, the supernatants were collected and stored at 20 ◦C. Supernatants were tested in ELISA assay, as described above with minor modifications. Samples were serially diluted starting from 1/2–

1/16 in two-fold dilutions in the blocking buffer (NFDM/T-TBS) and, after the washing step, the plates with the serial dilutions were incubated for 2 h at RT. After three washes, goat anti-mouse IgA Heavy Chain Antibody HRP (Bethyl Laboratories) diluted 1:5000 was added to each well and the plates were incubated at RT for 1 h. A cut-off value was determined for each plate by multiplying by three the average of the blank OD signal derived from wells not containing murine sample [28].

ELISpot assay Splenocytes were isolated from the murine spleen by gently pressing it through a 70 µm cell strainer using a syringe plunger. Cells were washed with RPMI-1640 medium with L-glutamine and 25 mM HEPES (Gibco) supplemented with 10% FBS (Euroclone) and 100X Penicillin/ Streptomycin (Euroclone) and centrifuged at 1300 rpm for 10 min. Red blood cells were lysed in 10X RBC Lysis buffer (BioLegend) and centrifuged at 1300 rpm for 10 min and the pellet was resuspended in FBS with 7.5% Dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored in the liquid nitrogen tank until use. The T cell responses of immunized mice were analyzed using a mouse IFN-γ/IL-4 Double-Color ELISPOT kit (CTL ImmunoSpot) and a pre-coated mouse TNF-α Single-Color ELISPOT kit (CTL ImmunoSpot), following the manufacturer's protocol. Briefly, the day before starting the assay, membrane-bottomed 96well plates were coated with anti-IL-4 and IFN-γ monoclonal capture antibodies and kept at 4 ◦C overnight, while the membrane-bottomed 96-well plate for TNF-α was ready-to use. The day after, 3 × 105 cells/ well splenocytes were seeded and stimulated in triplicate with the PepMixTM SARS-CoV-2 Spike Glycoprotein (1 µg/ml, JPT); complete CTL medium alone and phorbol myristate acetate (PMA)/Ionomycin (Invitrogen) were used as negative and positive control, respectively. The plates were incubated for 24 h in a humidified 5% CO2 incubator at 37 ◦C. Following incubation, plates were washed and biotinylated IL-4, TNF-α and IFN-γ antibodies were added for 2 h at RT. Finally, streptavidin-alkaline phosphatase was added and after 1 h of incubation substrate solutions were added. The reaction was stopped by extensively washing the plates with tap water, then the plates were left to dry at least 24 h in darkness. Wells were imaged with ImmunoSpot® Analyzers (CTL-Immunospot) and spot-forming units (SFUs) were determined with the ImmunoSpot® Double and Single Color Enzymatic Software Suite (CTL-Immunospot).

Micro-neutralization assay

The micro-neutralization (MN) assay was performed, as previously reported by Manenti and colleagues [30]. In brief, VERO E6 C1008 cells (CRL-1586) were cultured in Dulbecco's Modified Eagle's Medium (DMEM), High Glucose (Euroclone), supplemented with 2 mM L-glutamine (Lonza), 100 units/ml Penicillin–Streptomycin mixture (Lonza) and 10% Fetal Bovine serum (FBS) (Euroclone), in a 37 ◦C and 5% CO2 humidified incubator. Serial two-fold dilution of murine serum samples, starting from 1:10, were incubated with an equal volume of SARS-CoV-2 (Wuhan Strain) viral solution containing 25 tissue culture infective dose 50% (TCID50) for 1 h at RT [31]. After incubation, 100 µl of the serum-virus mixture was transferred to a 96-well plate containing an 80% sub-confluent Vero E6 cell monolayer containing 2% FBS. The plates were incubated for three days at 37 ◦C and 5% CO2. At the end of incubation, the presence/absence of cytopathic effect (CPE) was assessed by means of an inverted optical microscope. A CPE higher than 50% was indicative of infection. The MN titre was expressed as the reciprocal of the highest serum dilution showing protection from viral infection and CPE.

Histopathological examination

At sacrifice, mice underwent complete necropsy, and any gross change was recorded. The following organs/tissues were sampled and fixed in 10% neutral buffered formalin for histological examination: liver, kidneys, spleen (when available), lungs, lumbar skin and subcutis, inguinal lymph nodes, para-aortic lymph nodes, mesenteric lymph nodes, ileum-ceco-colic junction, and any other organ/tissue with grossly detectable lesions. Tissues were routinely processed for paraffin embedding, and 4 µm sections from each paraffin block were stained with hematoxylin-eosin (H&E) and evaluated under a light microscope. Findings were recorded according to the following grading system: 0 =absent; 1 =minimal; 2 =slight; 3 =moderate; 4 =marked; 5 =severe. Additionally, the number of intestinal lymphoid structures (part of the gut-associated lymphoid tissue, GALT) (small < 0.5 mm in diameter; medium: 0.5–2 mm in diameter; large > 2 mm in diameter) were recorded per each examined mouse. The total number of structures and the GALT total score (no. of small structures*1 + no. of medium structures*2 + no. of large structures*3) were then calculated. For the digital image analysis, digital slides were obtained from 4 µm H&Estained sections of the ileum-cecum-colon portion by using the NanoZoomer-XR Digital slide scanner (Hamamatsu, C12000), and images were captured by using the NDP.view2 Viewing software (Hamamatsu, U12388–01). The area of each lymphoid structure identified in the intestinal mucosa was measured using the freehand region

selection tool of the NDP.view2 Viewing software. The mean area of lymphoid structures and the total area were then calculated.

Statistical analysis

Statistics analyses were performed only for data collected within the framework of the main experiment, whereas, due to the limited sample size, only a qualitative assessment was performed for the preliminary experiment.

To investigate the effect of different immunization treatments on IgG levels, we relied on gamma generalized linear mixed models (GLMM) with log-link function, accounting for heterogeneity of variances in IgG values between time points (days 21, 35 and 48 post-vaccination) and experimental groups (listed in Table 2). Mouse identity was included as a random intercept effect to account for repeated measures of the same individual across time points, whereas experimental groups, time point, and their interaction were included as fixed factors. The effects of immunization treatments on IgA levels at day 48 post-vaccination were assessed by gamma generalized linear models (GLM) accounting for heterogeneity of variances among experimental groups. Separate models were fitted for treatment groups subjected to subcutaneous or rectal vaccine administration. For both IgG and IgA levels, significance of main effects was tested by Wald $\chi 2$ tests. Post-hoc pairwise comparisons were computed (on the log-scale) to investigate differences in IgG/ IgA values between experimental groups (within each time point for IgG models), adjusting p-values according to the Tukey method (on families of 5 estimates). Checks of model diagnostics (performed using the DHARMa R package, ver. 0.4.4) indicated that the models fitted the data adequately (details not shown for brevity).

The number of IFN-γ-, IL-4-, TNF-α-secreting cells were compared among mice from different treatment groups using Mann-Whitney tests. Because sample size was very small (< 5) for several groups (see Results), biologically relevant pairwise statistical comparisons between treatment groups were performed only when sample size for both groups was > = 5.

Variation between treatment groups in total number of lymphoid structures, GALT total score and total area of lymphoid structures in the intestinal tract of R vaccinated mice was assessed by Kruskal-Wallis tests. In case the Kruskal-Wallis test highlighted significant variation among groups, subsequent pairwise comparisons were performed by Mann-Whitney tests.

Variation in body mass among experimental groups from the main experiment between two time points (day 7 and day 42 postvaccination) was assessed by means of linear mixed models

(LMM), fitted separately for treatment groups subjected to subcutaneous or rectal vaccine administration. Mouse identity was included as a random intercept effect to account for repeated measures of the same individual across time points, whereas experimental groups, time point, and their interaction were included as fixed factors. Significance of fixed effects was assessed by F-tests, with degrees of freedom estimated according to the Kenward-Roger approximation. Post-hoc pairwise comparisons were computed to investigate differences in body weight between experimental groups (within each time point), adjusting p-values according to the Tukey method (on families of 5 estimates).

Gamma GL(M)Ms were fitted using the GLMMtmb R package (ver. 1.1.3, [32]), while LMMs were fitted using the lme4 R package (ver. 1.1–26, [33]). Pairwise comparisons were performed using the emmeans and multcomp R packages (ver. 1.7.1 and 1.4, respectively). All statistical analyses were performed using the R software (ver. 4.0.4; [34]).

Results

LeCoVax-2 vaccine design and production

The two clones of *L. tarentolae* (Lt-spike and Lt-RBD) expressing the full-length (1240 amino acids) SARS-CoV-2 virus spike protein sequence or the RBD-SD1 domain (273 aminoacids), were selected for the successive phases of the study. Before preparing the vaccine doses, the production of both proteins by the recombinant clones of *Leishmania* was verified by Western blotting, which allowed to specifically stain two bands, at approximately 180 and 35 kDa; this confirms the expression of the whole spike and of the RBD (Fig. 1 C; Supplementary Fig. 1–2). In addition, as shown in Fig. 1D, the presence of the spike protein on the membrane of *Leishmania* was revealed by immunofluorescence; the yellow/orange dots indicate the colocalization of the spike protein and the external membrane of the protozoan parasite, while green dots indicate that the spike protein was present also in the cytoplasm (see also [26]). Prior to mice immunizations with the different candidate vaccine formulations, spike-protein producing *Leishmania* cells were inactivated with formalin. As reported in Fig. 1E, the treatment did not alter the morphology of *Leishmania* cells; in fact, the parasites possessed an intact membrane and maintained their structure (Fig. 1E).

Mouse IgG responses to preliminary immunization

To determine the immunogenicity of the candidate vaccines, three groups of BALB/c mice were intraperitoneally immunized, and three groups of mice were subcutaneously administered with different compositions, without adjuvants.

Supplementary File 1 reports the specific anti-RBD IgG signals observed in sera samples; for some samples belonging to the groups of mice immunized with LeCoVax-2, subcutaneously or intraperitoneally, or intraperitoneally immunized with 10 μg RBD, the OD value was high (more than 1), corresponding to a high concentration of anti-RBD specific antibodies, with a linear decrease upon dilution, indicating the stability of the signal. No specific antibodies were detected in animals immunized with Lt-P10 (control strain) or PBS. Moreover, the serum of one out five intraperitoneally LeCoVax-2 vaccinated mice that tested positive in ELISA assay, displayed neutralizing activity against SARSCoV-2 in in vitro assays, indicating the presence of neutralizing antibodies. No significant differences in animal body weight, between vaccinated groups and control groups, were observed, and no local inflammation response at the injection site or other adverse effects were observed from the start of the study until sacrifice (data not shown).

Subcutaneous and rectal administration induced RBD-specific IgG and IgA responses in vaccinated mice

To test the immunogenicity and the induction of a humoral immune response, BALB/c mice were subcutaneously or intrarectally primed on day 0 and boosted on days 21 and 35 with different vaccine preparations, as described in Table 2.

Serum samples were collected from mice before each immunization and the IgG levels were determined. IgG values showed a statistically significant differential variation across time points among different treatment groups (gamma GLMM, treatment × time point interaction, $\chi 2 = 107.2$, d.f. = 8, $p < 0.0001$). As shown in Fig. 2 A, mice groups that were subcutaneously immunized showed high levels of SARS-CoV-2 RBD specific IgG antibodies, with a noticeable increase of OD values after the boosts (Fig. 2). In detail, after 21 days, the mice group vaccinated with LeCoVax-2 plus AddaVax showed significantly higher levels of IgG antibodies compared to other vaccinated groups and the PBS control (gamma GLMM, post-hoc tests correcting for multiple comparisons, $p < 0.002$ for all comparisons). After the first and second boost (day 48), the IgG levels increased in the mice groups vaccinated with LeCoVax-2 with AddaVax and in mice immunized with recombinant RBD and the AddaVax or Alum adjuvants,

showing statistically significant differences compared to the control group (gamma GLMM, post-hoc tests correcting for multiple comparisons, $p < 0.0001$ for all comparisons) (Fig. 2 A). Among all treated groups, mice immunized with recombinant RBD plus AddaVax showed the highest levels of specific IgG antibodies (Fig. 2 A).

Similarly to subcutaneous administration, IgG values of intrarectally immunized mice showed a statistically significant differential variation across time points among different treatment groups (gamma GLMM, treatment × time point interaction, $\chi2 = 113.77$, d.f. = 8, $p < 0.0001$). As shown in Fig. 2 A, after R immunization, the highest levels of specific IgG antibodies were detected after the third administration; in particular, mice immunized with LeCoVax-2 showed significantly higher levels of IgG antibodies compared to the control group (gamma GLMM, post-hoc tests correcting for multiple comparisons, $p < 0.001$ for both comparisons) (Fig. 2 A). On the other side, mice intrarectally vaccinated with the purified RBD antigen plus R848 displayed only a limited IgG response compared to the control group (Fig. 2 A).

The ability of the candidate vaccines to induce mucosal IgA was evaluated by performing ELISA against RBD, collecting faeces from vaccinated mice at day 48. A statistically significant treatment effect was observed both in the subcutaneously and intrarectally vaccinated mice (gamma GLMs, $\chi2 = 37.6$, d.f. = 4, $p < 0.0001$ and $\chi2 = 23.5$, d.f. = 4, $p < 0.0001$, respectively). However, post-hoc tests (correcting for multiple comparisons) revealed only a non-significant increase of OD values in the mice group subcutaneously immunized with the RBD protein plus Alum compared to the control group (Fig. 2B). Furthermore, none of the pairwise comparisons between treatment groups subjected to rectal vaccination was statistically significant, although values for the LeCoVax-2 +R848 group were somewhat higher than values for other experimental groups.

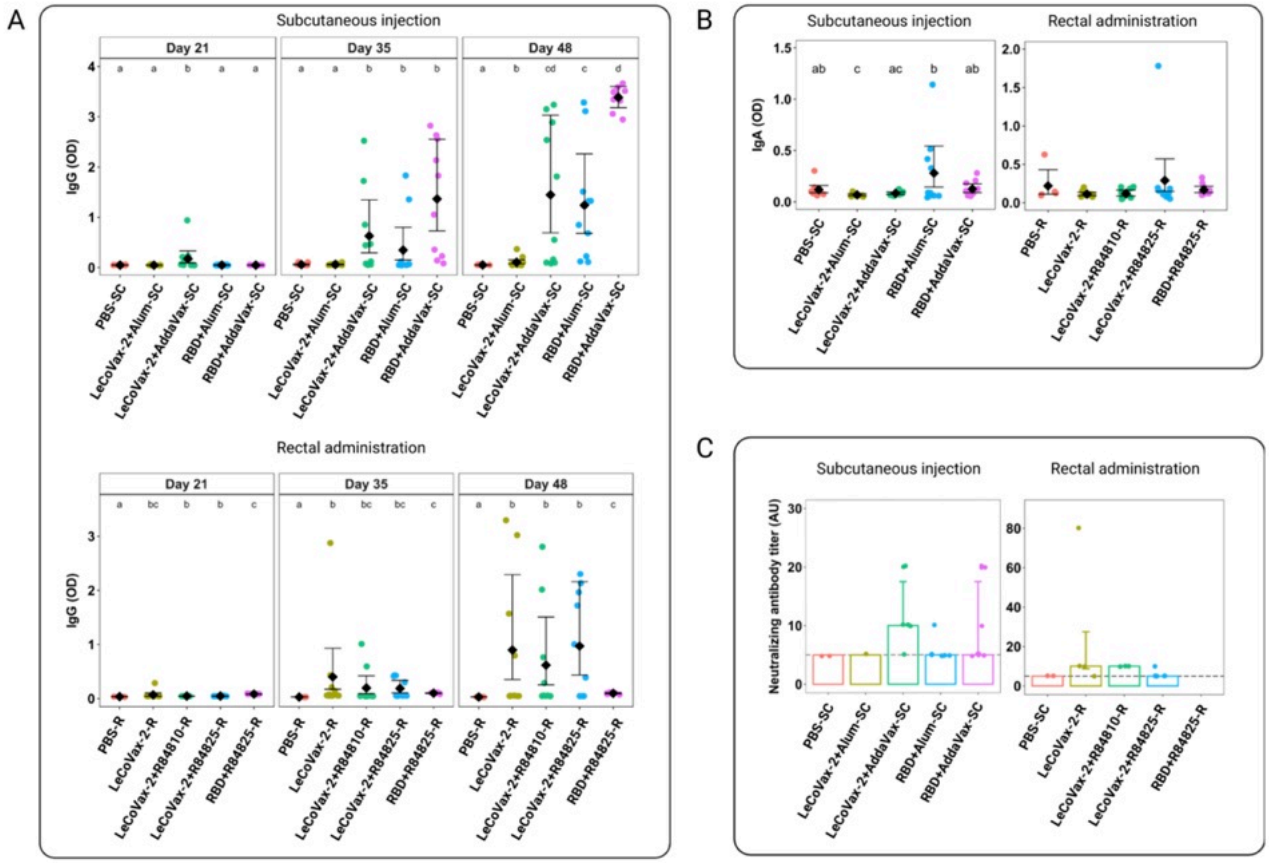

Fig. 2. Immune responses after immunization with LeCoVax-2 vaccine. (A-D) Antibody responses in sera of immunized mice were evaluated. (A) Variation of IgG levels detected at days 21, 35 and 48 and IgA levels at day 48 (B) after SC or R administration of different vaccine formulations in mice (PBS administered as control; n = 10 mice per experimental group, except for PBS-R, n = 5). Dots represent original data points, diamonds represent estimated mean values (with 95% CI) from a gamma GLMM accounting for heterogeneity of variances (see Methods). Different letters denote significant ($p < 0.05$) differences at post-hoc tests (after Tukey correction for multiple testing) within each time point (for IgA determination upon rectal administration, no pairwise comparison was significant after correction for multiple testing). (C) Neutralizing antibody titers at day 48 post-administration of vaccines (left: subcutaneous; right: rectal; note the different y-axis scales). Bars represent median values, error bars 25th and 75th percentiles. Random jittering was added to datapoint to show overlapping data. The horizontal dashed line represents the threshold over which samples were considered as the limit of detection (LOD). Antibody titers were determined only for samples that were IgG positive at day 48 post-administration. The PBS-SC group was included as a control for both subcutaneous and rectal administration; see Methods).

Antibodies induced by vaccination are capable of neutralizing SARSCoV-2

To evaluate viral neutralization capability, sera from vaccinated mice were tested in a MN neutralization assay. To this end, sera from immunized mice were assessed for their ability to prevent cytopathic effects of SARS-CoV-2 on Vero cells in vitro. Antibody titers, expressed as the reciprocal of the highest serum dilution showing protection from viral infection, were only determined for samples that were IgG positive at day 48.

Although no statistical comparison was performed because of the limited sample size for several experimental groups, as shown in Fig. 2 C and in Table 3, some animals which subcutaneously received LeCoVax-2 plus AddaVax or RBD plus AddaVax were able to neutralize the SARSCoV-2 with similarly high neutralization titers. Moreover, mice intrarectally immunized with LeCoVax-2 or LeCoVax-2 plus adjuvant (R848) were also able to neutralize the virus with higher neutralization titers, reaching values of up 1:80. Importantly, 75% of the mice that seroconverted to RBD, after immunization with both LeCoVax-2 or LeCoVax2 plus 10 μg R848 (overall, 6 out of 8 mice), displayed neutralizing activity to SARS-CoV-2 (Table 3). In contrast, no neutralizing capacity was detected in sera from non-vaccinated mice.

Formulation	IgG positive samples (Day 48)	SARS-CoV-2 neutralizing samples (Day 48)
LeCoVax-2 + AddaVax-SC	7/10 (70%)	5/7 (71%)
LeCoVax-2 + Alum-SC	2/10 (20%)	0/2 (0%)
20 μg RBD + AddaVax-SC	10/10 (100%)	4/10 (40%)
20 μg RBD + Alum-SC	8/10 (80%)	1/8 (13%)
LeCoVax-2 + 10 μg R848-R	4/10 (40%)	3/4 (75%)
LeCoVax-2 + 25 μg R848-R	6/10 (60%)	1/6 (17%)
LeCoVax-2-R	4/10 (40%)	3/4 (75%)
20 μg RBD + 25 μg R848-R	0/10 (0%)	0/10 (0%)
PBS-SC	0/10 (0%)	0/2 (0%)
PBS-R	0/10 (0%)	0/10 (0%)

RBD: Receptor Binding Domain; SC: subcutaneous; R: rectal; Alum: aluminium phosphate gel; AddaVax: squalene-based oil-in-water (w/o) nano-emulsion.

Table 3 IgG positive and SARS-CoV-2 neutralizing samples at day 48 in each tested group of the main experiment.

Vaccinated mice show antigen-specific T cell responses

RBD-specific T cell responses were measured through ELISpot analysis, using spleens from immunized mice after three vaccine doses. Quantification of IFN-γ-, IL-4-, TNF-α-secreting

cells after stimulation were determined and expressed as SFU/106 splenocytes. As reported in Fig. 3 A, splenocytes from mice vaccinated by SC injection with RBD plus adjuvants (AddaVax or Alum) and re-stimulated with RBD peptides yielded sensibly higher IL-4 secretion (compared to the control value), with significantly higher value in the RBD+AddaVax-SC group compared to LeCoVax-2 +AddaVax-SC (Mann-Whitney test, $p = 0.036$). For both IFN-γ and TNF-α-secreting T cells, no significant differences in response to RBD stimulation were obtained by analyzing the splenocytes derived from the same mice (Fig. 3 A). Only the group subcutaneously immunized with RBD plus Alum showed a statistically higher frequency of TNF-α-secreting cells compared to the group treated with RBD plus AddaVax (Mann-Whitney test, $p = 0.036$). Regarding the analyses of splenocytes derived from mice vaccinated through the R route, some secretion of TNF-α and IFN-γ was observed in groups treated with LeCoVax-2 plus adjuvant, compared with other groups or PBS treated mice. No IL-4 secretion was detected in vaccinated mice compared to PBS treated mice (Fig. 3B). We emphasize that T cell response was determined only on animals with positive IgG titers at day 48 and that statistical analyses on T cell responses were performed only for the groups for which the number of IgG-positive animals was ≥ 5 for both groups being compared in pairwise tests.

LeCoVax-2 does not induce adverse effects

Histological examination revealed that SC and R administration of the different formulations in BALB/c mice induced no adverse effects in liver, kidney, and lung. We observed the development of germinal centers in secondary lymphoid organs (spleen and LNs) as result of an antigenic stimulation; in particular, it was detected in periaortic LNs after SC and R administration of different tested formulations, and in one single spleen after R administration of LeCoVax-2 plus 25 μg R848. However, no obvious association with production of IgG or neutralizing antibody was observed. Histological and digital image analyses of the GALT revealed a significant variation in the number of lymphoid structures between groups (Kruskal-Wallis test, $\chi2 = 8{,}02$, d.f. = 3, $p = 0.045$) and a marginally non-significant variation in the GALT total score (Kruskal-Wallis test, $\chi2 = 7.06$, d.f. = 3, $p = 0.07$), whereas the GALT total area did not significantly vary among treatment groups (Kruskal-Wallis test, $\chi2 = 4.6$, d.f. = 3, $p = 0.21$) (Fig. 4 A). For the number of lymphoid structures, subsequent pairwise comparisons revealed that LeCoVax-2-R848 group showed significantly higher values compared to the control group (Mann-Whitney test, $p = 0.017$). Similarly, to the development of germinal centers observed in

LNs, no association between increase in GALT and production of IgG or neutralizing antibody was observed.

All the animals immunized with the different vaccine formulations were monitored daily for adverse events, including body weight loss. As shown in Fig. 4B, the body weight of the animals, both immunized subcutaneously and rectally, increased from the first week of observation to the last one before the sacrifice. The change in body weight was not statistically significant different between groups for subcutaneous administration (treatment × time point, $F_{4,45} = 0.83$, $p = 0.52$), whereas there were some differences between groups for the rectal administration ($F_{4,40} = 3.63$, $p = 0.013$). However, for the latter, posthoc pairwise comparisons revealed no significant differences in body weight between groups within each time point (all $p > 0.45$, Fig. 4B).

Discussion

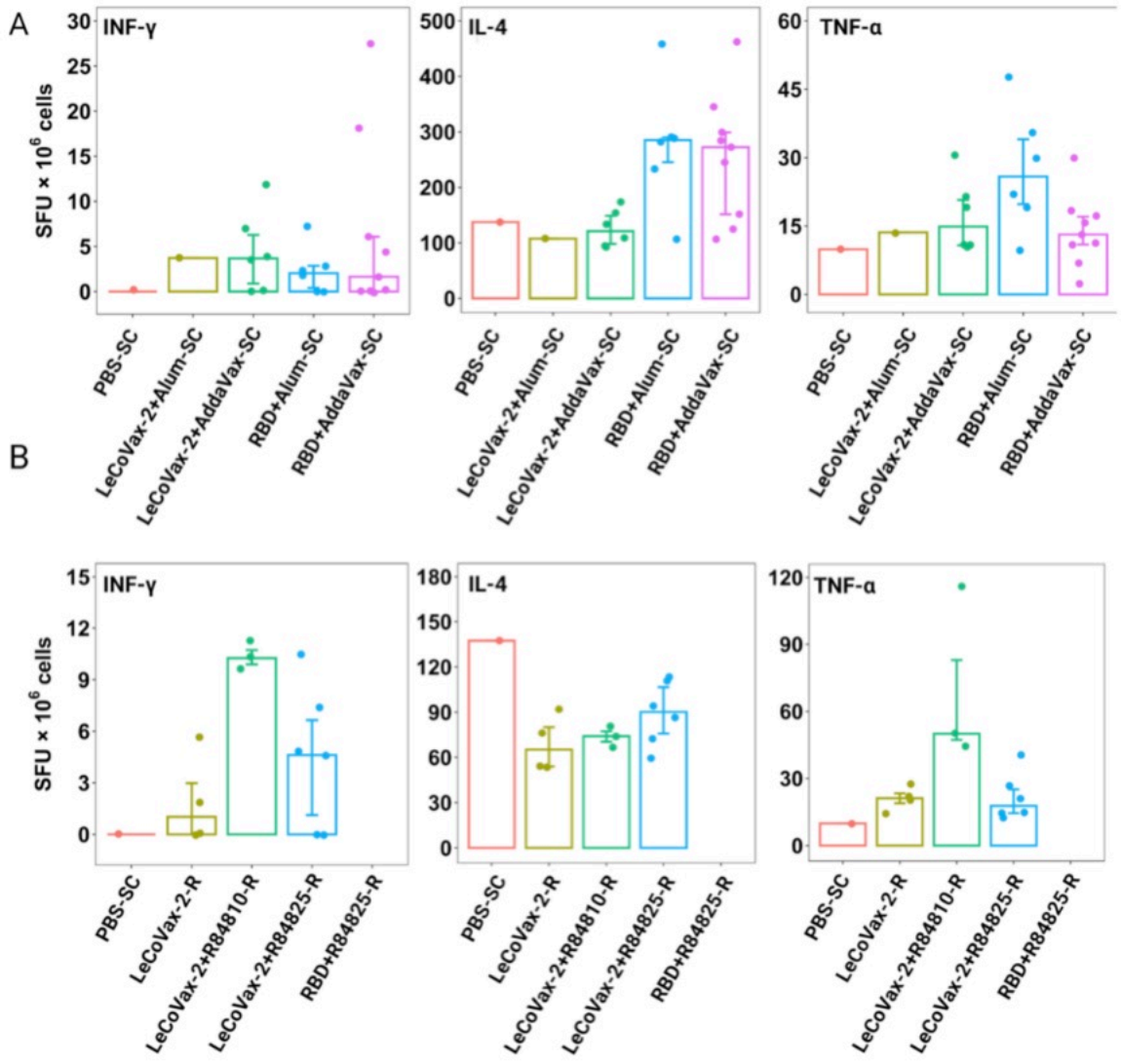

Fig. 3. Evaluation of antigen-specific cytokine-producing T cells capacity. The number of IFN-γ, IL-4 and TNF-α secreting cells per million splenocytes (spot forming units, SFU) was determined by ELISpot analysis at day 48 post-administration of LeCoVax-2 through the SC route (A) and R route (B). Only samples that were IgG positive at day 48 post-administration were evaluated. Bars represent median values, error bars 25th and 75th percentiles. For rectal administration, we lacked PBS treated individuals (see Methods): for comparison, we thus reported the values obtained from the single individual which received a SC injection.

The aim of the present study was to assess, as proof-of-principle, the potential of the parasitic protozoan *L. tarentolae* as a vehicle for enteral vaccination. To avoid the possible pitfalls associated with oral administration, we focused on the rectal route. Indeed, oral administration would have required ad hoc solutions to protect the vaccine vehicle and the antigen from gastro-duodenal digestion, which was out of the scope of this study. In the selection of SARS-CoV-2 as a virus model, we considered the still wide circulation of this virus in human populations, and the possibility that additional booster vaccine doses could still be required in the future, for the control of COVID-19 pandemic. In this context, a vaccine preparation suitable for mucosal administration may represent an important alternative to current types of vaccines. Several COVID-19 vaccine candidates for nasal or inhalation delivery are currently being tested and have already proved effective in determining a mucosal IgA-based immunity, in addition to cellular immunity and IgG-based humoral responses [1,35]. The rectal route remains however to be investigated, and our study presents a first example of a candidate COVID-19 vaccine tested in rectal administration. Notably, antigen administration through the enteral route can stimulate B-cell clones that home to the respiratory apparatus, in addition to the intestine [36,37]. This would thus guarantee specific mucosal responses, at both the gut and respiratory levels.

In addition to the use of *L. tarentolae* as a vaccine platform, a further peculiarity of our study is that we assayed a preparation (LeCoVax-2), containing both *L. tarentolae* expressing the spike protein (Lt-spike) and the purified RBD domain of this protein, produced by another engineered clone of the protozoon. The rationale of this strategy is to immunize the host with both a subunit antigen (the recombinant RBD) as well as with a particulate microbial antigen (Lt-spike, where the spike protein includes the RBD domain). The particulate Lt-spike component of LeCoVax-2 is expected to be internalized with high efficacy by DCs [25,26], and then processed and

presented to T CD4 + lymphocytes, which should result in a more effective immune response [38].

Our study consisted of two experiments in the murine model. In the first, preliminary experiment, three types of antigen preparations (Ltspike; RBD; LeCoVax-2) were administered without adjuvants. As detailed in the results section, IgG response without adjuvants was limited, and was evident only after the third administration (second booster dose). Moreover, only one of the 30 animals vaccinated with the three antigen preparations, at the different dosages and combinations, developed a neutralizing response. Despite the failure in term of the production of a neutralizing response, this first experiment provided encouraging results, and useful information for the prosecution of the study: 1) none of the animals, at any antigen dosage and combination, displayed any detectable adverse reaction; 2) the potential of *L. tarentolae* as a vaccine platform and vehicle was confirmed, considering the generation of the IgG response even in the absence of adjuvants; 3) the LeCoVax-2 preparation was well tolerated even after SC inoculation of 2 × 107 Lt-spike *Leishmania* cells in combination with 10 µg of purified RBD antigen, or after IP administration.

In the second experiment, we introduced three types of adjuvants and the rectal route of administration (while the IP route was not further assayed, considering its limited potential in terms of medical translation). Comparison with the results of the first experiment demonstrates that even a vaccine vehicle such as L. *tarentolae*, which is expected to target DCs and secondary lymphoid tissues [26], benefits from co-administration with adjuvants in SC administration. Indeed, some animals in different groups seroconverted even after the first booster dose, and the percentage of seropositive animals increased after the second boost (while in the first experiment, in the absence of adjuvants, most of the response was observed only after the second boost). Considering the neutralizing properties of the sera from SC immunized animals, the AddaVax adjuvant appears more effective than Alum, in agreement with ELISA assay data assessing IgG responses.

The most important result of the second experiment was the seroconversion in animals immunized through the rectal route. Indeed, while seroconversion after SC antigen administration was not surprising, particularly in the presence of adjuvants, the rectal route for antigen administration has, to date, only been explored in a limited number of studies. In addition, *Leishmania* or any other Trypanosomatidae species have never been investigated as potential rectal vaccines or vaccine vehicles, against COVID-19 or other pathogens. Comparing

the results of SC and rectal antigen administration, while in SC administration the purified RBD antigen, co-administered with AddaVax, was very effective in determining seroconversion, in rectal administration this purified antigen failed to induce any significant IgG production. On the contrary, the LeCoVax-2 preparation, which includes *Leishmania* cells expressing the spike antigen, determined significant seroconversion after rectal administration. As for the results of the viral neutralization assays, almost all groups produced sera that neutralized SARS-CoV-2, albeit with varying percentages of neutralizing animals in the different groups (see Table 3). We emphasize that the number of neutralizing animals in SC administration was notably higher after the use of AddaVax as adjuvant, as compared to alum-adjuvanted animals (e.g., see Table 3, groups 2 and 4). In the case of rectal delivery, the percentage of neutralizing animals was variable (see discussion below), but the administration of the LeCoVax-2 preparation did not appear to differ in efficacy after administration with or without the mucosal adjuvant R848.

As already emphasized, a rather notable result that we observed after rectal administration was the absence of any detectable IgG responses induced by the purified RBD antigen, which was in contrast very effective in determining the IgG response after SC administration. This contrasts with the results obtained using LeCoVax-2, which was very effective in rectal administration, even in the absence of adjuvants. To summarize, while in SC administration the use of an adjuvant is required for the generation of a significant IgG response (consider the limited and delayed IgG response observed in the first, non-adjuvanted, experiment), in rectal administration the presence of the adjuvant does not appear to be required. In addition, while in SC administration the response induced by LeCoVax-2 (which include Lt-spike cells) did not differ significantly from that induced by the purified RBD antigens, in rectal administration we did not observe a significant response after the use of the purified RBD antigen alone, even in the presence of the adjuvant. The fact that the response to RBD was observed, in rectal administration, only when Lt-spike cells were present (i.e., in the LeCoVax-2 preparation) provides strong evidence for the role played by *Leishmania* cells in the generation of the immune response.

A major problem that we encountered during our experiments was that some mice defecated within a relatively short time after rectal administration of the antigen preparations. For logistic reasons we could not keep the mice under observation for over 20 min after rectal vaccination, but the fact that one to three mice per group expelled a fecal pellet within this short time suggests that the retention time of the antigen preparation (or part of it) in the mice gut could

have been on average rather short, possibly limited to a few tens of minutes, with unpredictable variation of retention times among the animals. In addition, some leakage of the preparation from the anus cannot be excluded. This could possibly explain the variability of the results obtained after rectal administration, and the limited response in terms of IgA production. More in general, both oral and rectal drug administration are expected to imply rather variable interindividual adsorption of the active compound [39,40]. We should also consider that the gavage we used for rectal administration was calibrated to ensure the delivery of the preparation in the upper part of the colon, without reaching the ileo-cecal valve (to avoid the risk of determining any lesion). Therefore, we assume that the antigen had not been delivered into the small intestine, and did not reach the ileum, where most Peyer's patches are located.

Considering the limitations of rectal antigen administration, particularly in animal models, where defecation cannot be controlled, we believe that the variations we observed within and between the groups, in terms of IgG and neutralizing response, do not affect the importance of the results that we obtained with the candidate vaccine LeCoVax-2, in terms of a future development of *L. tarentolae* as a vehicle for mucosal vaccination (either rectal or oral). For example, LeCoVax-2, or fractions of *Leishmania* cells loaded with the desired antigen, could be prepared as tablets for oral administration, and coated for protection against gastric digestion and for a controlled release in the distal part of the small intestine. This would ensure a higher retention time of the antigen in the gut, compared to the time ensured by rectal administration, and the potential translocation of the antigen to lymphoid cells not only in the colon, but also in the ileum and through the M cells and the Peyer's patches. Therefore, besides the possibility that our study could offer clues for the development of future strategies for the administration of booster doses in anti-COVID-19 vaccination, our results are of more general interest, and provide a strong a case on the potential of L. *tarentolae*, not only as a platform for vaccine production, but also as a vehicle for antigen delivery through the enteral route.

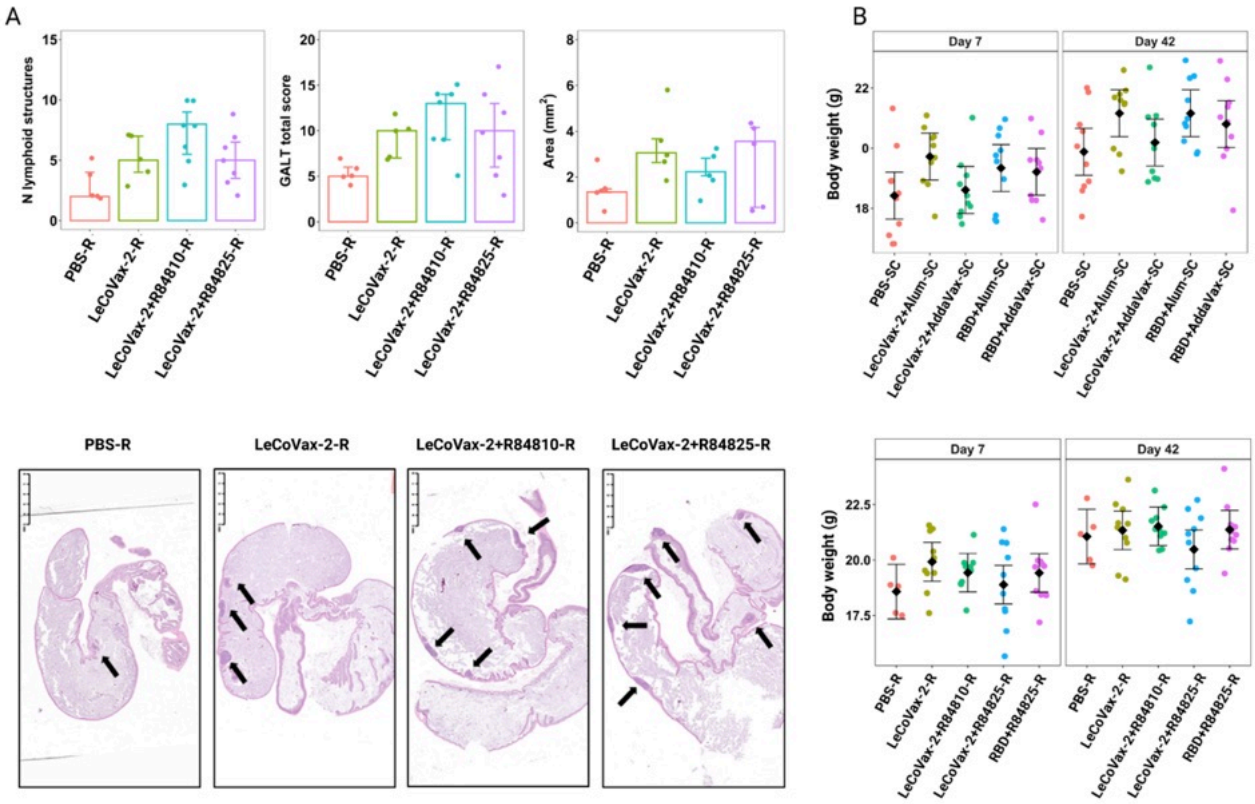

Fig. 4. Histological analyses for adverse effects determination. (A) Total number of lymphoid structures, GALT total score and total area of lymphoid structures in the intestinal tract of R-vaccinated mice in proximity of the ileo-cecal-colic junction are shown. Box plots represent median, 25th and 75th percentiles, minimum and maximum values. Arrows indicate lymphoid structures of gut-associated lymphoid tissue. (B) Body weight variation of mice subjected to subcutaneous (upper panel) or rectal (lower panel) administration of different vaccine formulations (including PBS administration as a control group) in mice (n = 10 mice per experimental group, with the exception of PBS-R, n = 5). Dots represent original datapoints, diamonds represent estimated mean values (with 95% CI) from a LMM (see Methods). No pairwise comparison (within each time point) was significant upon correction for multiple testing.

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CRediT authorship contribution statement

C.B., S.E., P.F. E.M. and GV.Z.: Conceptualization. I.V.B., S.E., G.C., F.D., M.M., I.R, M.L., L.G., L.B.: Data collection. C.B., S.E., A.M., D.R., P. G., C.R., M.I.: Data analysis and interpretations. C.B., GV.Z.: Supervision. I.V.B., S.E., L.M.: Methodology. C.B., S.E., GV.Z., E.M.: Resources. C.B., S.E., I.V.B.: Writing – original draft. L.G., P.F., A.M., D.R.: Critical revision of the manuscript.

Declaration of Competing Interest

The candidate vaccine Lt-spike and its potential application have been described in the PCT/IB2022/051585 (23 February 2022). The antigen Lt-RBD and its potential application have been described in the patent application no. IT 02021000004160 (23 February 2021). A.M., F. D., M.M., and I.R. are employed by VisMederi Srl; M.L. is employed by VisMederi Research Srl; E.M. is the President and Founder of VisMederi Group. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The data that support the findings of this study are included in this published article (and its supplementary information files).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2022.106546.

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7.1.3 Rectal Administration of *Leishmania* Cells Elicits a Specific, Th1-Associated IgG2a Response in Mice: New Perspectives for Mucosal Vaccination against leishmaniasis, after the Repurposing of a Study on an Anti-Viral Vaccine Candidate

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Abstract

The mucosal immune system plays a pivotal role in the control of infections, as it represents the first line of defense against most pathogens, from respiratory viruses to intestinal parasites.

Mucosal vaccination is thus regarded as a promising strategy to protect animals, including humans, from infections that are acquired by ingestion, inhalation or through the urogenital system. In addition, antigens delivered at the mucosal level can also elicit systemic immune responses. Therefore, mucosal vaccination is potentially effective also against systemic infections acquired through non-mucosal routes, for example, through the bite of hematophagous insects, as in the case of leishmaniasis, a widespread disease that affects humans and dogs. Here, we explored the potential of antigen rectal administration for the generation of anti-*Leishmania* immunity. Mice were immunized through rectal administration of whole cells of the model parasite *Leishmania tarentolae* (using a clone engineered to express the spike protein of the SARS-CoV-2 virus generated in a previous study). A specific anti-*Leishmania* IgG antibody response was detected. In addition, the recorded IgG2a/IgG1 ratio was higher than that of animals injected subcutaneously; therefore, suggesting a shift to a Th1-biased immune response. Considering the importance of a Th1 polarization as a protective response against *Leishmania* infections, we suggest that further investigation should be focused on the development of novel types of vaccines against these parasites based on rectal immunization.

Keywords: mucosal immunity; canine leishmaniasis; sera repurposing; *Leishmania* vaccine; Th1/Th2 immune polarization; LeCoVax-2

Introduction

Leishmania spp. are a group of protozoa infecting several vertebrate species, including humans. Major diseases caused by these microorganisms comprise visceral leishmaniasis in humans and a multisystemic pathology in dogs, known as canine leishmaniasis [1,2]. Despite decades of research efforts in *Leishmania* vaccinology, none of the numerous candidate vaccines tested has been translated into products suitable to be used in large-scale vaccination campaigns in humans, and capable of determining high-level protection, associated with persistent immunity [3]. Obstacles towards developing effective *Leishmania* vaccines stem, at least in part, from the type of immunity required to achieve an effective protection against the disease [3,4]. Indeed, anti-*Leishmania* immunity should develop on the Th1 side, with classical M1 macrophage activation, and limited Th2 response, with low production of antibodies [4–7]. Indeed, an excess in non-protective antibody production in

chronic leishmaniasis is associated with immune-complex disease and should ideally be avoided. In this context, adjuvants are of great importance, since molecules that polarize the response on the Th1 side are expected to favor the development of a protective response, while those biassing the response on Th2 could potentially worsen the clinical course of the disease [7,8]. Consequently, most of the investigations in *Leishmania* immunology and vaccine development have been focused on factors involved in immune-modulation, chiefly on adjuvants polarizing the response on the Th1 side. In contrast, the effects of the route of vaccine administration on the polarization of the immune response have generally been overlooked, in *Leishmania* infections as well as in other diseases. Considering the practical advantages of this type of needle-less delivery of the antigens (see Conclusion), and of the role of mucosa-associated lymphoid tissues in the modulation of both local and systemic immunity, mucosal vaccination is worthy of further investigations. Indeed, gut immunity is now regarded as a major player in immune modulation [9,10]. Vaccine administration through the enteral route could thus be exploited also for its potential to modulate the immune response on the desired direction. Here, we investigated whether rectal administration of *Leishmania* parasites is suitable to elicit a specific immune response, and whether this response develops towards the desired Th1 side. To this end, we repurposed groups of sera obtained in the context of a previous study that exploited the model parasite *Leishmania tarentolae*, as a vaccine platform in anti-viral vaccination (see details in Materials and Methods) [11,12].

Materials and Methods

Clones of L. tarentolae

In a previous study, *L. tarentolae* was engineered to express the spike protein antigen from SARS-CoV-2 [13]. The engineered clone of *L. tarentolae* (Lt-spike) was then used to prepare a candidate anti-COVID-19 vaccine that was tested in immunization assays in mice. The formulation used in mice immunization contained 108 whole cells of the Lt-spike clone and the purified receptor binding domain (RBD) fragment, which is a portion of the spike protein. The combination of Lt-spike plus purified RDB was named LeCoVax-2. This previous study was designed to investigate the immune response of mice against SARS-CoV-2; therefore, the antibody response against *L. tarentolae* had not been determined.

Mice Immunization

To perform this study, we used sera samples derived from a previous experiment ([13]; authorizations: D4A18.B.1YW; D4A18.B.FX6), in which female BALB/c mice were immunized with LeCoVax-2. The animals were used according to Directive 2010/63/UE regarding the protection of animals used for experimental or other scientific purposes, enforced by the Italian Legislative Decree n◦ 26 of 2014 and Ministerial. Mice were rectally immunized on day 0, 21 and 35 with different formulations as follows: (i) LeCoVax-2 (Lt-spike and recombinant RBD antigen); (ii) LeCoVax-2 mixed with 10 µg Resiquimod (R848, Invivogen, San Diego, CA, USA); (iii) LeCoVax-2 mixed with 25 µg Resiquimod; (iv) PBS as placebo. Mice were also immunized subcutaneously with LeCoVax-2+ AddaVax (Invivogen, San Diego, CA, USA); sera from these mice were included in this study as a reference group, for comparison with intrarectal administration. Sera were collected on day 0 (prior to immunization), 21, 35 and 48 for the characterization of the antibody response (Table 1).

Experimental Group	Formulation	IgG1 *Leishmania* Positive Samples	IgG1 SARS-CoV-2 Positive Samples	IgG2a *Leishmania* Positive Samples
Subcutaneous injection				
LeCoVax-2 + AddaVax-SC	2×10^7 cells Lt-spike + 10 µg RBD + AddaVax	5/5	3/5	5/5
Rectal administration				
PBS-R	PBS solution (control)	0/5	0/5	0/5
LeCoVax-2-R	1×10^8 cells Lt-spike + 20 µg RBD	4/10	4/10	4/10
LeCoVax-2 + R848 10-R	1×10^8 cells Lt-spike + 20 µg RBD + 10 µg R848	2/10	4/10	4/10
LeCoVax-2 + R848 25-R	1×10^8 cells Lt-spike + 20 µg RBD + 25 µg R848	4/10	6/10	6/10

Lt: *Leishmania tarentolae*; RBD: Receptor Binding Domain; SC: subcutaneous; R: rectal; AddaVax: squalene-based oil-in-water nano-emulsion; R848: Resiquimod.

Table 1. Details of experimental groups and of IgG-positive samples at day 48 in each tested group.

In-House Enzyme-Linked Immunosorbent Assay (ELISA)

Preparation of Leishmania tarentolae Antigen (LtAg)

L. tarentolae (P10 strain) promastigotes were cultured in brain heart infusion (BHI) liquid medium supplemented with 5 µg/mL porcine hemin, 50,000 U/L penicillin, 50 mg/L streptomycin (Jena Bioscience, Jena, Germany) at 26 ◦C in the dark under aerated conditions. *L. tarentolae* promastigote antigens (LtAg) were prepared following the protocol described in [14]. Briefly, *L. tarentolae* cells were washed twice in phosphate buffered saline (PBS), then the pellet was lysed with a buffer containing 50 mM Tris, 5 mM EDTA and protease inhibitors

(Invitrogen, Waltham, MA, USA) and then subjected to three rapid freeze/thaw cycles followed by six sonication pulses of 20 s/40 W. Then, the samples were centrifuged at 10,000× g for 20 min at 4 ◦C and the supernatants were collected and stored at −80 ◦C until use. The protein concentration was determined with the Nanodrop spectrophotometer.

Set Up of In-House Leishmania IgG ELISA Assay

An in-house IgG ELISA assay was set up using LtAg as the coating antigen for the detection of specific *Leishmania* antibodies in the sera of murine samples. A series of preliminary assays was performed to select the optimal antigen concentration (0.5, 1, 1.5, 3, 4 µg/mL) testing five murine sera collected from BALB/c mice immunized via subcutaneous route with *L. tarentolae* wild type promastigotes (strain P10) and obtained with the preliminary study described in [13]; these sera were previously tested in immunofluorescent assay and resulted positive for *Leishmania* antibody response. Plates were coated with the five different LtAg concentrations and incubated overnight at 4 ◦C. After three washes with tris-buffered saline (TBS)–0.05% (v/v) Tween 20 (TBS-T), plates were blocked for 1 h with TBS-T containing 5% (w/v) non-fat dry milk (NFDM; Euroclone, Pero, Italy). Subsequently, two-fold serial dilutions, starting from 1:100 in blocking buffer, were performed and then 100 µL of each serial dilution was added for 1 h at 37 ◦C. Then, goat anti-mouse IgG1 HRP-conjugated (Bethyl Laboratories, Montgomery, TX, USA) diluted at 1:50,000 was added for 30 min at 37 ◦C. Finally, 3,3',5,5'-tetramethylbenzidine substrate (Sigma Aldrich, St. Louis, MO, USA) was added for 20 min and then HCl solution 0.5 N to stop the reaction (Fisher Chemical, Waltham, MA, USA).

Detection of Leishmania IgG1 and IgG2a in Murine Sera

The four animal groups and the control group are presented in Table 1. Sera collected from each animal and at each time point were tested in ELISA assay to investigate the presence of specific IgG *Leishmania* antibodies. The assay was performed as described above according to the following optimized parameters: 3 µg/mL of LtAg as coating concentration, isotype-specific HRP-conjugated secondary antibodies diluted both 1:50,000 (goat anti-mouse IgG1, Bethyl Laboratories, Montgomery, TX, USA or goat anti-mouse IgG2a, Abcam, Cambridge, UK) and sera two-folded diluted starting from 1:50 in blocking buffer. A cut-off value was obtained for each plate by multiplying by three the average of the blank optical density (OD) signal derived from wells not containing the analyte (background) [15].

Statistical Analysis

Variation in IgG1 and IgG2a levels among the five experimental treatments across the three time points (day 21, 35 and 48 post-vaccination) was assessed by means of gamma generalized linear mixed models (GLMMs) with log-link function, including experimental (5-level fixed factor; see Table 1), time point (3-level fixed factor) and their interaction as predictors. Mouse identity was included as a random intercept effect. Fitted models accounted for heterogeneity of variances in IgG values between time points (IgG1) and experimental groups (IgG2a). To assess variation in Th1 polarization among vaccinated groups of mice, we fitted a linear model of the IgG2a/IgG1 ratio at day 48 post-vaccination including the experimental group as a 4-level factor (control mice excluded). Post hoc pairwise comparisons were computed to investigate differences in IgG levels or IgG2a/IgG1 ratio between experimental groups (within each time point for gamma GLMMs of IgG1 and IgG2a), adjusting p-values according to the Tukey method (on families of 5 or 4 estimates, respectively). The association between anti-*Leishmania* IgG1 and anti-RBD IgG1 antibody levels assayed in the same individuals (n = 40) was assessed by the Spearman correlation coefficient. Gamma GLMMs were fitted using the glmmTMB R package (ver. 1.1.3). Pairwise comparisons were performed using the emmeans and multcomp R packages (ver. 1.7.1 and 1.4, respectively). All statistical analyses were performed using the R software (ver. 4.0.4; [16]).

Results and Discussion

The purpose of the current study was to explore whether rectal administration of whole, inactivated cells from *L. tarentolae* is suitable to induce a specific antibody response against *L. tarentolae* itself. To perform this study, we took advantage from a previous study, in which whole cells of *L. tarentolae* (from the clone Lt-spike) had been administered to BALB/c mice through the rectal route, with the purpose of assaying a candidate vaccine against COVID-19 (see [13] and Material and Methods section). The aim of this previous study was to determine the response against SARS-CoV-2; determination of anti-*Leishmania* antibody production was thus not performed. Sera from this previous work had been stored at -80 C; we thus retrieved the sera and determined the presence of antibodies against *L. tarentolae* using an in-house ELISA assay, also typing the two main IgG subclasses, IgG1 and IgG2a.

Set Up of In-House ELISA Assay

Lt-Ag, the antigen prepared from *L. tarentolae* promastigotes, was tested for its ability to detect specific *Leishmania* IgG antibodies in the sera of immunized mice. Of the four antigen concentrations assayed, we finally chose a concentration of 3 µg/mL for the coating of ELISA plates. Among the five positive sera used to set up the ELISA assay, the serum with the highest OD value at the first dilution was chosen as positive control to be used in the following test (Tables S1 and S2).

Anti-Leishmania IgG Antibody Response in Mice after Rectal Immunization, and Correlation with the IgG Response against SARS-CoV-2

Table 1 presents the groups and animals, and the details of the preparations used in immunization. The IgG1 and IgG2a responses against *L. tarentolae* are reported in Figure 1. IgG1 values showed a statistically significant differential variation across time points among different formulations (gamma GLMM, treatment × time point interaction, $\chi 2 = 35.8$, d.f. = 8, $p < 0.0001$), with an increase of OD values mostly after the second administration (Figure 1A). After 21 days, mice vaccinated subcutaneously with LeCoVax-2 plus AddaVax showed significantly higher levels of IgG1 antibodies compared to other vaccinated mice and controls ($p < 0.001$ for all comparisons). The same trend was observed after 35 days, while after the third administration (i.e., after day 48), a higher number of positive mice was scored in all experimental groups (Table 1). As reported in Table 1, mice immunized subcutaneously with LeCoVax-2 plus AddaVax developed anti-*Leishmania* antibodies with statistically significant differences compared to the control group ($p < 0.0001$), as well as to groups that received intrarectal vaccination, with LeCoVax2 ($p < 0.05$), LeCoVax-2 + R84810 ($p < 0.001$) and LeCoVax-2 + R84825 ($p < 0.05$). In addition, mice immunized through the rectal route with LeCoVax-2 + R84825 also showed statistically significant differences compared to the controls ($p < 0.05$) (Figure 1A). Interestingly, mice that showed antibodies against *Leishmania* were the same that produced antibodies against SARS-CoV-2 in the previous study (Table 1) [13], except for two animals (from the subcutaneous group) that developed only antibodies against *Leishmania*. Overall, a positive correlation between the two responses (i.e., against SARS-CoV-2 and *Leishmania* spp.) was observed (Spearman's rank correlation $\rho = 0.70$; $p < 0.0001$). This positive correlation suggests that variability of the response of mice in the groups intrarectally immunized may be due to the characteristics of the route of administration. As discussed in

[13], rectal vaccination implies that the retention of the antigen in the intestine is variable, in some way unpredictable, and this can explain the variability of the results obtained after vaccination through this route.

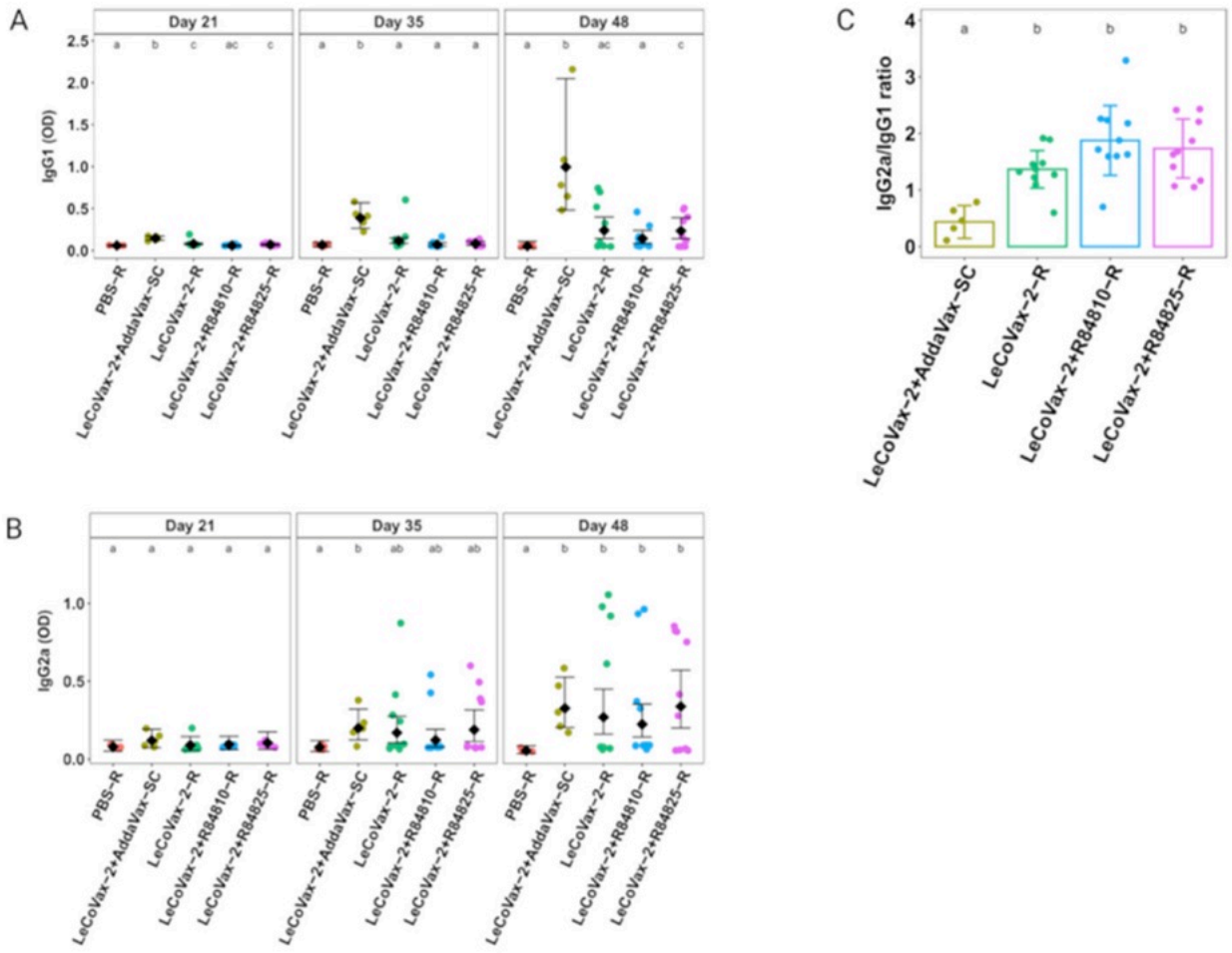

Figure 1. IgG1 and IgG2a antibody response after rectal immunization with LeCoVax-2. (A,B) Variation of IgG1 and IgG2a levels detected at days 21, 35 and 48 after subcutaneous or rectal administration of different formulations of LeCoVax-2 in mice (n = 10 mice per experimental group, except for PBS-R, n = 5). Dots represent original data points; diamonds represent estimated mean values (with 95% CI) from gamma GLMMs accounting for heterogeneity of variances (see Section 2). (C) IgG2a/IgG1 ratio at day 48 after subcutaneous or rectal administration of the different formulations in mice (bars represent mean values; error bars represent standard deviation). In all plots, in the upper part of each graph, different lowercase letters indicate significant ($p < 0.05$) differences at post hoc tests (after Tukey correction for multiple testing) (within each time point for (A,B)). For example, label 'ac' denotes a statistically

significant difference compared to groups labeled as ‘b’, but a non-significant difference compared to groups ‘a’ and ‘c’.

Specific IgG Subtype Responses for the Different Routes of Administration

The specific IgG2a immune response against *Leishmania* was assessed by ELISA assay and the IgG2a/IgG1 ratio was then calculated. A statistically significant differential variation of IgG2a across the three time points among different formulations (gamma GLMM, treatment × time point interaction, $\chi2 = 87.7$, d.f. = 8, $p < 0.0001$) was found, with an increase of OD values starting from the second dose of the treatment (day 35). After the first immunization, no significant differences were detected in the groups, while after 35 days, an increasing number of positive animals in all immunized groups was recorded, showing a statistically significant difference between mice vaccinated with LeCoVax-2 + AddaVax and mice of the control group ($p < 0.05$) (Figure 1B). After the third administration (48 days), the highest number of positive mice was detected in all groups, with statistically significant differences with the PBS control group (for all comparisons $p < 0.0001$ except for LeCoVax-2 and LeCoVax-2 + R84810: $p < 0.001$) (Figure 1B).

The IgG2a/IgG1 ratio, a marker associated with the polarization of the immune response on the Th1 or Th2 sides [17], revealed that mice receiving a subcutaneous administration showed a IgG2a/IgG1 ratio lower than 1 (value = 0.43), suggesting a shift towards a Th2 response; conversely, mice intrarectally immunized showed a higher IgG2a/IgG1 ratio, over 1.5, indicating a Th1-biased immune response (linear model, effect of treatment group: $\chi2 = 37.94$, d.f. = 3, $p < 0.0001$). The differences were statistically significant for all three groups intrarectally immunized compared with subcutaneously immunized mice (LeCoVax-2: $p < 0.01$; LeCoVax-2 + R84810: $p < 0.0001$; LeCoVax-2 + R84825: $p < 0.001$) (Figure 1C). These results agree with those of previous studies on other infectious agents, showing that the subcutaneous route of antigen administration is more likely to induce a Th2 immune response compared with rectal administration, which is commonly associated with a more balanced response, or with a shift on the Th1 side [18–20]. The highest IgG2a/IgG1 ratio was observed in mice intrarectally immunized with LeCoVax-2 with the lower dose of adjuvant (value = 1.9). Furthermore, intrarectal administration of LeCoVax-2 determined a Th1-biased response against *L. tarentolae* also in the absence of any adjuvant. The possibility to induce a correctly polarized

immune response against *Leishmania* spp., in the absence of adjuvant, further emphasizes the potential of rectal vaccination for developing effective leishmaniasis vaccines.

Conclusions

Enteral vaccination presents both drawbacks and advantages compared to other modes of antigen delivery [21]. A first advantage is that enteral vaccination, similarly to other forms of mucosal vaccinations, is potentially suitable to elicit an immune response at the mucosal level, with production of secretory IgA antibodies. A second advantage is the possibility to exploit the modulation of the immune response, which is one of the functions of gut-associated lymphoid tissues (GALT). Indeed, a major role of GALT is the interplay with food-derived molecules and with the microbiota, in the modulation and balancing of the overall functioning of the immune system [9,10]. Finally, preparation of vaccines for enteral vaccination is facilitated by the lower requirements in terms of purity and sterility, compared with classic vaccines, e.g., those for intramuscular administration. So far, a sole study investigated the potential of enteral vaccination against *Leishmania*, using a murine model. In this study, a cell lysate of *Leishmania* amazonensis was administered orally; parameters of the immune modulation were then determined, with evidence for a specific, Th1-biased polarization of the response [22]. Besides this study, which dates to 2003, all successive investigations on mucosal vaccination in *Leishmania* spp. have exploited the nasal/inhalation route for the administration of the antigen (e.g., [23,24]). Overall, the results of our investigation on rectal administration of *Leishmania* antigens are coherent with those obtained after oral administration in the 2003 study, displaying a significant production of IgG antibodies against the administered *Leishmania* species, with a shift toward a specific Th1-associated IgG2a response. In conclusion, although in the present study we used an engineered strain of L. *tarentolae*, and this could have influenced the specific production of anti-*Leishmania* antibodies, considering the importance of Th1 polarization for a protective response against *Leishmania* spp. infections, and the advantages of mucosal vaccination, rectal immunization is worth of further investigation towards the development of novel vaccines against these parasites.

Patents

The antigen Lt-spike and its potential application in vaccination against coronaviruses have been described in the PCT/IB2022/051585 (23 February 2022). The antigen Lt-RBD and its potential application have been described in patent application no. IT 02021000004160 (23 February 2021).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/tropicalmed8080406/s1, Table S1. Plate layout and OD values of the set-up of the first ELISA assay. Table S2. Plate layout and OD values of the second set-up of the first ELISA assay.

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7.1.4 Intracellular persistence of *Leishmania tarentolae* in primary canine macrophage cells

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Abstract

Leishmania tarentolae is a non-pathogenic species first isolated from geckoes in the Mediterranean basin. The finding that dogs test positive against both *Leishmania infantum* and *L. tarentolae* raises questions regarding the ability of the latter species to persist and adapt to new hosts. This study aimed to evaluate in vitro the capability of *L. tarentolae* to colonize, survive and persist in canine primary monocyte-derived mononuclear cells. Monocytes were isolated from dog whole blood samples and placed in 24-well plates for differentiation into macrophages and for incubation with *L. tarentolae* field-isolated strains (RI-325 and SF-178) and laboratory (LEM-124) strain; the parasite burden was assessed at different time points post-infection. The *L. infantum* laboratory strain (MON1) was used as control. Infection parameters were evaluated by microscopy, counting the number of amastigotes/ 200 infected cells, and by duplex real-time PCR from supernatants and detached cells. Similar to *L. infantum*, *L. tarentolae* strains developed into round-shaped amastigote-like forms, with higher

infection rates detected at 4 h followed by an overall decrease until 48 h. RI-325 presented also a higher infection rate at 72 h. Data showed that *L. tarentolae* strains infect and persist inside in vitro primary canine mononuclear cells, opening new perspectives for further laboratory studies.

Introduction

*Leishmania*ses, caused by *Leishmania* spp. (Kinetoplastida, Trypanosomatidae), are neglected vector-borne tropical diseases that infect more than 350 million people worldwide (WHO, 2023). Although the infection mostly affects people from a poor socioeconomic context in East Africa, South-East Asia, and Latin America, the disease is also endemic in several Mediterranean countries (Pace, 2014; WHO 2023). The genus *Leishmania* comprises more than fifty species, which infect and replicate in dendritic cells (DC) and macrophages (Breton et al., 2005, 2007; Taylor et al., 2010; Varotto-Boccazzi et al., 2022). *Leishmania* infantum, causing visceral leishmaniasis, is the most widespread species (Morales-Yuste et al., 2022), being transmitted by sand flies of the genera Lutzomyia in the New World (Dantas-Torres et al., 2012) and *Phlebotomus* in the Old World (Maroli et al., 2013). Furthermore, the success of *Leishmania* spp. to infect different mammalian hosts (Mann et al., 2021) relies on the parasite's capacity to respond and adapt to changes in various host environments (Sp äth et al., 2015). This is the case of *Leishmania tarentolae* (subgenus *Sauroleishmania*), an herpetophilic species first detected in the gecko Tarentola mauritanica (Elwasila, 1988) and subsequently in Podarcis filfolensis and Podarcis siculus lizards (Mendoza-Roldan et al., 2021; 2022a). *Leishmania tarentolae* is vectored by Sergentomyia minuta, a herpetophilic sand fly (Mendoza-Roldan et al., 2021), which was previously reported to ingest human and dog blood (Abbate et al., 2020). Also, the high prevalence of *S. minuta* in countries of the Mediterranean basin (Otranto et al., 2007; Tarallo et al., 2010) suggests the existence of a wider host spectrum than previously believed (Maia and Depaquit, 2016), eventually explaining the detection of *L. tarentolae* DNA also in human and dog blood (Pombi et al., 2020; Iatta et al., 2021; Mendoza-Roldan et al., 2022b). In addition, *L. infantum* and *L. tarentolae* overlap in their geographical distribution, raising questions regarding the ability of the latter species to persist and adapt in non-conventional hosts (Mendoza-Roldan et al., 2022a). The capability of *Leishmania* spp. to infect mononuclear cells of different mammals (e. g., humans, dogs, mice) has been demonstrated in a range of in vitro studies using cultured promastigotes (Maia et al., 2007). The

demonstration of *L. tarentolae* uptake by mouse and human macrophages and dendritic cells coupled with intracellular development, also in human cells (Breton et al., 2005; Taylor et al., 2010; Varotto-Boccazzi et al., 2022), opens the exciting possibility that these parasites may be immunogenetic and thus represent potential, live-attenuated vaccine candidates (Epis et al., 2022; Bandi et al., 2023).

However, although *L. tarentolae* infection has been documented in intraperitoneal murine macrophages and THP-1 human cultured cell lines (Breton et al., 2005; 2007), no information is available on how *L. tarentolae* survives inside dog macrophages, even though these parasites may exert possible protection against canine leishmaniasis (Mendoza-Roldan et al., 2022a). Thus, this study aimed to evaluate the capability of *L. tarentolae* to infect, survive and persist in primary canine monocyte-derived mononuclear cells.

Materials and methods

Animal source

A one-year-old male crossbreed dog, after a written informed consent obtained from the owner, was used as a source of whole blood and serologically tested for leishmaniasis by indirect immunofluorescent antibody test (IFAT), and molecularly by duplex real-time PCR (dqPCR) as previously described (Iatta et al., 2021; Latrofa et al., 2021). The study protocol was approved by the ethical committee of the Department of Veterinary Medicine of the University of Bari, Italy (Prot. Uniba 21/2022). The methods were carried out in accordance with international, national, and/or institutional guidelines and regulations for handling animal cells.

Parasites and cultures

Leishmania strains were cryopreserved at 80 ◦C and tested as detailed in the following. The laboratory (RTAR/IT/81/ISS21-G.6c/ LEM-124) and field-isolated strains of *L. tarentolae* respectively isolated from T. mauritanica gecko (RTAR/IT/21/RI-325) and *S. minuta* (ISER/ IT/21/SF-178) were tested. An *L. infantum* zymodeme MON-1, previously typed by the Istituto Superiore di Sanita` (Rome, Italy), was used as a control. All the above strains were cultured in 1 ml of blood agar and Tobie-Evans, supplemented with Fluorocytosine (250 µg/mL), Gentamicin (250 µg/mL), and Fetal Bovine Serum (FBS, 5%). Cultures were maintained at 26 ◦C for five days before cell infection.

Preparation of primary canine monocyte-derived mononuclear cells

Mononuclear cells from peripheral blood were obtained according to Berman et al. (1979) protocol with modifications. Briefly, 10 ml of dog blood was collected in Ethylene Diamine Tetra Acetic acid (EDTA) tubes (BD Vacutainer, San Diego CA, USA) mixed with the same volume of RPMI-1640 medium (Euroclone, Milan, Italy), and centrifugated with a double volume of Lympholyte®-H (Cedarlane, Italy) at 400 x g for 45 min. The obtained buffy coat was washed twice with 15 ml of warm RPMI-1640 medium by centrifugation at 310 x g and 210 x g for 10 min, respectively. The obtained peripheral blood mononuclear cells were suspended in a warm RPMI-1640 medium supplemented with FBS (10%) and a mix of antibiotics (100X of Penicillin-Streptomycin, and 250 µg/mL of Amphotericin B). Cells at a concentration of 2 × 106/ml were added in each well of a 24-well plate containing sterile coverslips, in which approximately 10% maturates in macrophages. Cells were plated into wells without coverslips for use in dqPCR analyses. The plates were incubated at 37 ◦C with 5% CO2 for 24 h (h) for the adhesion of the mononuclear cells. Subsequently, the medium was replaced to remove non-adherent cells, and 1 ml of fresh RPMI-1640 supplemented with 10% of FBS and 10% of antibiotics mix was added. The plate was incubated for 120 h at 37 ◦C with 5% CO2 for complete macrophage maturation.

Infection of primary canine monocyte-derived mononuclear cells

Promastigotes from *L. tarentolae* (LEM-124, RI-325, and SF-178) and *L. infantum* (MON-1) cultures at the stationary phase of growth were collected and centrifuged at 210 x g for 10 min and washed thrice with sterile PBS (1X). The final pellet was resuspended in RPMI-1640 and counted in a Burker chamber at a dilution of 1:100. Promastigotes of each strain at a concentration of 2 × 106 parasites/ml (parasite/ macrophage ratio 10:1) were added to the plate after medium removal, and subsequently incubated at 37 ◦C with 5% CO2. Each well was washed thrice with sterile PBS after 4 h of infection to remove noninternalized parasites, and the plates were maintained to assess the internalization capacity over the post-infection time points. The supernatants and scraped cells from each timepoint of infection were collected and stored at 20 ◦C until DNA extraction. For each time point, two coverslips containing infected macrophages were stained with DiffQuick® (Merck, Darmstadt, Germany) following the standard protocol (Skipper and Stephano, 1989) or subjected to IFAT. Coverslips were fixed for 30 min in acetone (80%) and subsequently incubated with serum (dilution 1:200) of *L.*

tarentolae positive dog for 30 min at 37 ◦C. After three washes with PBS, the conjugate Anti-Dog IgG secondary antibody (Sigma-Aldrich, Germany) at 1:50 was added and coverslips were incubated for 30 min at 37 ◦C. The washes were repeated and the slides were observed under fluorescence microscopy at 100X (Zeiss Axio Imager.M2, excitation wavelength of 385 nm) by the incorporation of Evans Blue dye (1:500) to evidence the cell nucleus (red) from the *Leishmania* parasites (green). Controls were tested with serum from a *L. infantum*-positive dog. All experiments were performed in triplicates.

Evaluation of Leishmania spp. internalization and persistence

The internalization capacity of *Leishmania* parasites within macrophage cells was assessed at 2 and 4 h of incubation, while the survival and persistence were estimated after the following time points (8, 24, 48, 72, and 96 h) (Tables 1, 2). Stained cells on coverslips were observed under bright-field microscopy at 100X magnification (LEICA DM LB2, Germany). The internalization capacity of parasites was evaluated by stipulating the percentual of infected cells, the number of amastigotes in 200 infected cells (a/infc), and by quantification of *Leishmania* spp. DNA by dqPCR using the Cycle Threshold (Ct) value. Genomic DNA was extracted from scraped cells and supernatants using QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amastigotes' morphology was monitored by evaluating the size and shape during the time points of infection.

Statistical data

Statistical analyses were performed using GraphPad Prism v. 8.0.0 (GraphPad Software, San Diego, CA, USA) and data were reported as mean ± Standard Deviation (SD) for microscopical parameters and by the medium Ct value for dqPCR quantifications. Shapiro–Wilk test was used to check normal data distribution. Two-way ANOVA followed by Tukey's post hoc test was performed for the triplicates of each variable. Values were considered statistically significant when $p < 0.05$.

Infection (hours)	Laboratory strain				Field-isolated strains							
	LEM-124				RI-325				SF-178			
	Infected cells mean (%) ± SD	Amastigotes in infected cells (a/infc) mean ± SD	dqPCR Infected cells (Ct)	dqPCR Supernatant (Ct)	Infected cells mean (%) ± SD	Amastigotes in infected cells (a/infc) mean ± SD	dqPCR Infected cells (Ct)	dqPCR Supernatant (Ct)	Infected cells mean (%) ± SD	Amastigotes in infected cells (a/infc) mean ± SD	dqPCR Infected cells (Ct)	dqPCR Supernatant (Ct)
0	–	–	–	15.94^{a}	–	–	–	18.46^{a}	–	–	–	15.57^{a}
2	48.6 ± 2.60^{a}	$1.26 \pm 0.12^{a,b}$	19.10^{a}	16.13^{a}	50.4 ± 1.41^{a}	1.39 ± 0.09^{a}	20.85^{a}	$17.75^{a,b}$	39.8 ± 1.83^{a}	1.58 ± 0.07^{a}	23.28^{a}	17.47^{b}
4	58.9 ± 2.57^{b}	1.56 ± 0.04^{c}	16.78^{b}	17.04^{a}	55.5 ± 0.78^{b}	1.74 ± 0.04^{b}	18.37^{b}	16.86^{b}	44.8 ± 2.01^{b}	1.88 ± 0.07^{b}	21.21^{b}	23.97^{c}
8	41.6 ± 2.73^{c}	1.34 ± 0.04^{a}	$20.77^{a,c}$	19.35^{b}	47.7 ± 0.72^{a}	1.63 ± 0.06^{b}	21.27^{a}	21.22^{c}	22.6 ± 1.15^{c}	$1.50 \pm 0.03^{a,c}$	$24.43^{a,c}$	22.75^{c}
24	34.1 ± 2.61^{d}	$1.24 \pm 0.06^{a,d}$	$22.50^{c,d}$	22.65^{c}	38.6 ± 1.95^{c}	1.37 ± 0.06^{a}	24.60^{c}	22.72^{c}	$20.7 \pm 1.16^{c,d}$	$1.20 \pm 0.04^{d,e}$	$24.97^{a,c,d}$	$24.18^{c,d}$
48	26.5 ± 0.68^{e}	1.06 ± 0.02^{d}	$24.12^{d,e}$	20.95^{d}	32.6 ± 1.65^{d}	1.35 ± 0.22^{a}	27.64^{d}	25.23^{d}	$17.3 \pm 0.85^{d,e}$	1.07 ± 0.02^{d}	$26.05^{c,d}$	$25.57^{d,e}$
72	28.1 ± 0.60^{e}	$1.09 \pm 0.01^{b,d}$	25.80^{e}	22.80^{c}	37.7 ± 1.30^{c}	1.09 ± 0.05^{c}	25.48^{c}	24.60^{d}	15.8 ± 0.41^{e}	$1.31 \pm 0.02^{c,e}$	26.75^{d}	27.01^{e}
96	29.5 ± 0.71^{e}	$1.19 \pm 0.08^{a,d}$	24.37^{e}	25.22^{e}	26.3 ± 1.85^{e}	1.32 ± 0.07^{a}	29.49^{e}	26.16^{d}	$17.2 \pm 0.35^{d,e}$	$1.15 \pm 0.06^{d,e}$	$25.39^{c,d}$	26.49^{e}

Table 1 Infection data according to different time points for *Leishmania tarentolae* strains with the determination of the Cycle Threshold (Ct) by duplex real-time PCR (dqPCR) and microscopical rates of infection, expressed in percentage (%) and the medium number of amastigotes inside infected cells (a/200infc). LEM-124 = Laboratory strain; RI-325 = gecko strain; SF-178 = sand fly strain. The significative differences ($p<0.05$) between timepoints on each infection parameters for each strain were calculated by ANOVA two-way using GraphPad Prism 8. Different letters indicate significative differences. Same letters indicate no significative difference.

Infection (hours)	Laboratory strain			
	Leishmania infantum (MON-1)			
	Infected cells mean (%) ± SD	Amastigotes in infected cells (a/infc) mean ± SD	dqPCR Infected cells (Ct)	dqPCR Supernatant (Ct)
0	–	–	–	17.73^{a}
2	48.5 ± 1.49^{a}	$1.35 \pm 0.05^{a,b}$	21.19^{a}	$18.60^{a,b}$
4	54.4 ± 1.52^{b}	2.02 ± 0.11^{c}	19.34^{b}	19.37^{b}
8	37.1 ± 1.45^{c}	$1.28 \pm 0.07^{a,d}$	24.30^{c}	23.94^{c}
24	30.9 ± 1.35^{d}	$1.41 \pm 0.17^{a,b,d}$	27.57^{d}	$25.90^{d,e}$
48	27.6 ± 0.88^{d}	1.31 ± 0.05^{a}	29.61^{e}	$24.45^{c,d}$
72	19.3 ± 0.65^{e}	1.22 ± 0.04^{a}	30.94^{e}	26.35^{e}
96	23.4 ± 1.05^{f}	1.56 ± 0.14^{b}	27.57^{d}	28.29^{f}

Table 2 Infection data at different timepoints for *Leishmania* infantum strain (MON-1), with the determination of the Cycle Threshold (Ct) by duplex real-time PCR (dqPCR) and microscopical rates of infection, expressed in percentage (%) and the number of amastigotes inside infected cells (a/infc/200). The significative differences ($p<0.05$) between time points on each infection parameters were calculated by ANOVA two-way using GraphPad Prism 8. Different letters indicate significative difference. Same letters indicate no significative difference.

Results

Promastigotes of all *Leishmania* strains developed as amastigote forms inside primary canine monocyte-derived macrophage cells (Fig. 1), as observed under optical microscopy. *Leishmania tarentolae* amastigotes' internalization was observed also by immunofluorescence (Fig. 1F) at different time points. The intracellular amastigote's morphology remained round-shaped after 96 h, lacking an external flagellum, and measuring approximately 2 to 3 µm in diameter. Intraand extracellular parasite growth was monitored by microscopical cell counting and dqPCR (Fig. 2, Table 1). A statistically significant increase (Fig. 2A-B, Table 1) was observed in the infection rates by cell counting (ranging from 44.8% to 58.9%) and by dqPCR for scraped cells (Ct value ranging from 16.78 to 21.21) for all *L. tarentolae* strains at 4 h compared to 2 h post-infection, followed by a constant decrease until 48 h (up to 17.3%; Ct value of 26.05). In addition, the RI-325 gecko strain presented a statistically significant increase in the infection rate at 72 h (37.7%; Ct = 25.48), followed by a statistically significant decrease at 96 h (26.3%, Ct = 29.49) (Fig. 2A-B, Table 1). The average number of amastigotes of *L. tarentolae* strains inside each infected cell (a/infc) significantly increased (Fig. 2C) until 4 h (ranging from 1.56 to 1.88 a/infc, $p \leq 0.0008$), followed by a decrease until 48 h (ranging from 1.06 to 1.35 a/ infc). A statistically significant increase of a/infc was observed for RI325 (from 1.09 to 1.32 a/infc, $p = 0.0170$) at 96 h and for SF-178 at 72 h (from 1.07 to 1.31 a/infc, $p = 0.0179$) (Fig. 2C, Table 1). The analyses of the supernatant by dqPCR were in accordance with the pattern of a/infc with Ct values ranging from 16.86 to 26.49 at 4 h and 96 h, respectively (Fig. 2D, Table 1).

A similar picture was observed for *L. infantum* MON-1 analyses, with statistically significant increases at 4 h on the infection rate by microscopy (54.4% and 2.02 a/infc) and by dqPCR (Ct = 19.34), followed by an overall decrease until 72 h (19.3%, 1.22 a/infc, Ct = 30.94). A statistically significant increase was observed on the a/infc at 96 h (23.4%, 1.56 a/infc; Ct = 27.57, $p = 0.0001$) (Fig. 2, Table 2).

Discussion

Data indicate that *L. tarentolae* may infect and persist in primary canine macrophage cells, as inferred by amastigote cell infection rates and parasite loads. Furthermore, the reduction of *Leishmania* DNA in the supernatant could be indicative of the occurrence of promastigote internalization. The higher infection rate of *L. tarentolae* RI-325 observed at 72 h (37.7%) in relation to the LEM-124 strain (28.1%), probably due to constant parasite uptake of

promastigotes that could remain after washing the cells, suggests that the field-isolated strain could be more virulent than the laboratory one, even if the number of a/ infc decreases. Accordingly, previous observations indicated that the in vitro virulence of *Leishmania* spp. decreases through the passages in culture medium (Bussotti et al., 2018).

Moreover, a reduction of the infection rate detected for all *L. tarentolae* strains from 4 h to 48 h, with an increase for RI-325 at 72 h, was in agreement with data observed in mice primary macrophage cells infected by L. *donovani* (Baek et al., 2020). Indeed, in the latter study, a similar pattern was observed with a decrease in the infection rate from 3 h (80%) to 48 h (70%), followed by an increase at 72 h (80%) (Baek et al., 2020).

Furthermore, the increase in infection rate for the RI-325 gecko strain at 72 h differed from that observed for *L. tarentolae* (TARII) laboratory stain in human cells (THP-1), where a constant decrease was consistently recorded until 96 h post-infection (Breton et al., 2005). The differences in cell infectivity patterns could be due to virulence factors (i.e., amastigote-specific protein A2) of laboratory strains (TARII), as an effect of accumulation of mutations and loss of genes during the culturing (e.g. in the mitochondrial genome) (Raymond et al., 2012; Klatt et al., 2019). In fact, a more recently isolated *L. tarentolae* gecko strain (i.e., LEM-125) showed a higher ability to infect and develop in amastigotes forms than TARII in human cells (i.e., U-937, and macrophages derived from peripheral blood monocytes) (Taylor et al., 2010; Klatt et al., 2019). Similarly, the ability of *L. tarentolae* field-isolated strain to infect human dendritic cells were observed under experimental conditions (Varotto-Boccazzi et al., 2022), with an infection rate of 43% at 4 h, decreasing to 20% after 48 h.

Overall, all the above suggests that *L. tarentolae* is able to infect primary canine monocyte-derived macrophage cells with a reduction in amastigote number (up to 1.15 a/infc at 96 h), potentially due to an active immune cellular responsiveness. This reduction could also be due to a non-conventional host cell type to maintain the infection, as previously assumed for mice cells infected by *L. tarentolae* (Breton et al., 2005).

Nonetheless, the susceptibility of the primary canine monocytederived macrophage cells to *L. tarentolae* infection was supported by similar results herein observed for *L. infantum*, with higher values at 4 h (54.4%) and lowest at 72 h (19.3%). This data overlap those previously described for an *L. infantum* strain isolated from a naturally infected dog, in which a decrease in the infection rate was observed from 2 h (76%) to 96 h (5%), in primary canine cells (Tunon et al., 2015). Otherwise, in commercial canine macrophage cells (i.e., DH82), different infection

rates (from 50% and 60% after 24 h) were detected, indicating a different susceptibility to infect primary or commercial canine cells (Maia et al., 2007).

The infection and survival of *L. tarentolae* on primary canine monocyte-derived macrophages herein detected until 96 h could justify the molecular and serological in vivo detection of this protozoan parasite in naturally infected dogs (Mendoza-Roldan et al., 2021). In addition, data suggest that *L. tarentolae* may persist in canids, further emphasizing its potential usefulness as a vaccine target (Bandi et al., 2023), and on its protective effect in dogs when co-infected with *L. infantum*.

Overall, these results are of potential importance for studying the relationship between *L. tarentolae* and mammalian hosts through cytokine expression, as well as the interactions with *L. infantum* and resistance mechanisms. Moreover, the successful evidence of *L. tarentolae* infection in canine primary macrophage cells, its high genomic similarity with other pathogenic species (i.e., *L. infantum* and *Leishmania* major), and the absence of virulence factors further emphasize the utility of this parasite to be assayed as a future vaccine against canine leishmaniasis.

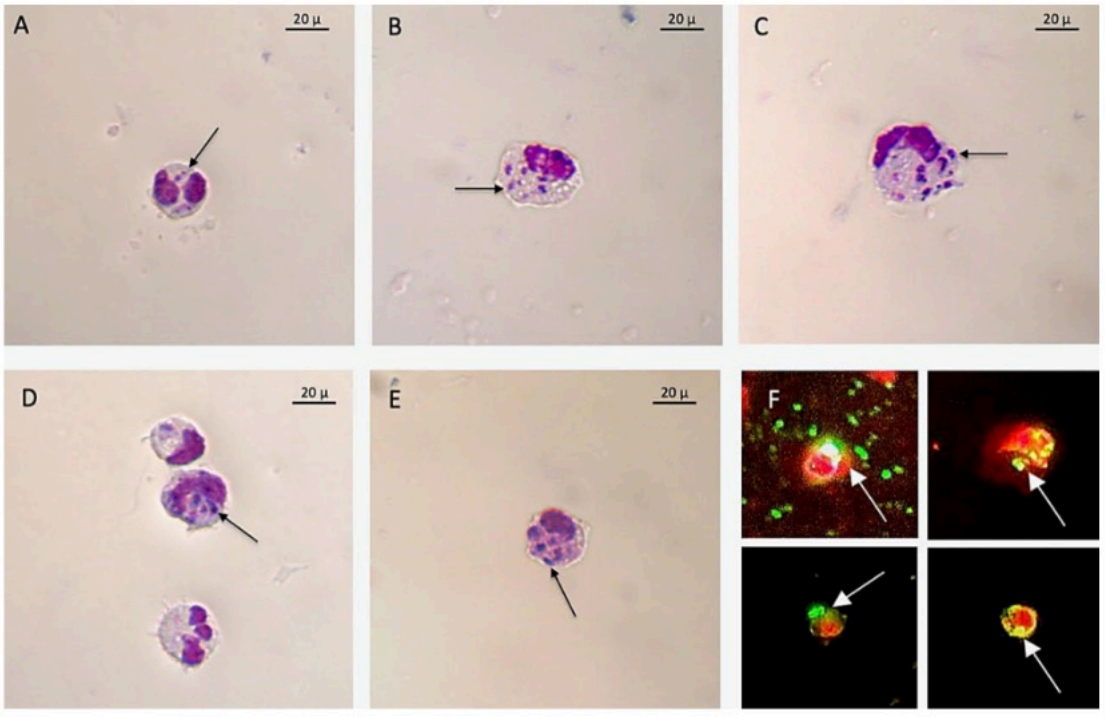

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Fig. 1. Diff-Quik smears of primary canine monocytic-derived macrophage cells infected by *L. tarentolae* LEM-124 after 4 h of infection (a); *L. tarentolae* RI-325 after 4 h of infection (b); *L. tarentolae* RI-325 after 72 h of infection (c); *L. tarentolae* SF-178 after 4 h of infection (d); and *L. infantum* MON-1 after 4 h of infection (e) under light microscopy. Black arrows indicate the

amastigote forms inside the cells. Immunofluorescence of primary canine monocytic-derived macrophage cells infected by *L. tarentolae* RI-325 strain at 4 h of infection (f), where *Leishmania* parasites are illustrated in green and cell nuclei are illustrated in red. White arrows indicate the amastigote forms inside the cells. *Leishmania* are illustrated in green; Cell nuclei are illustrated in red.

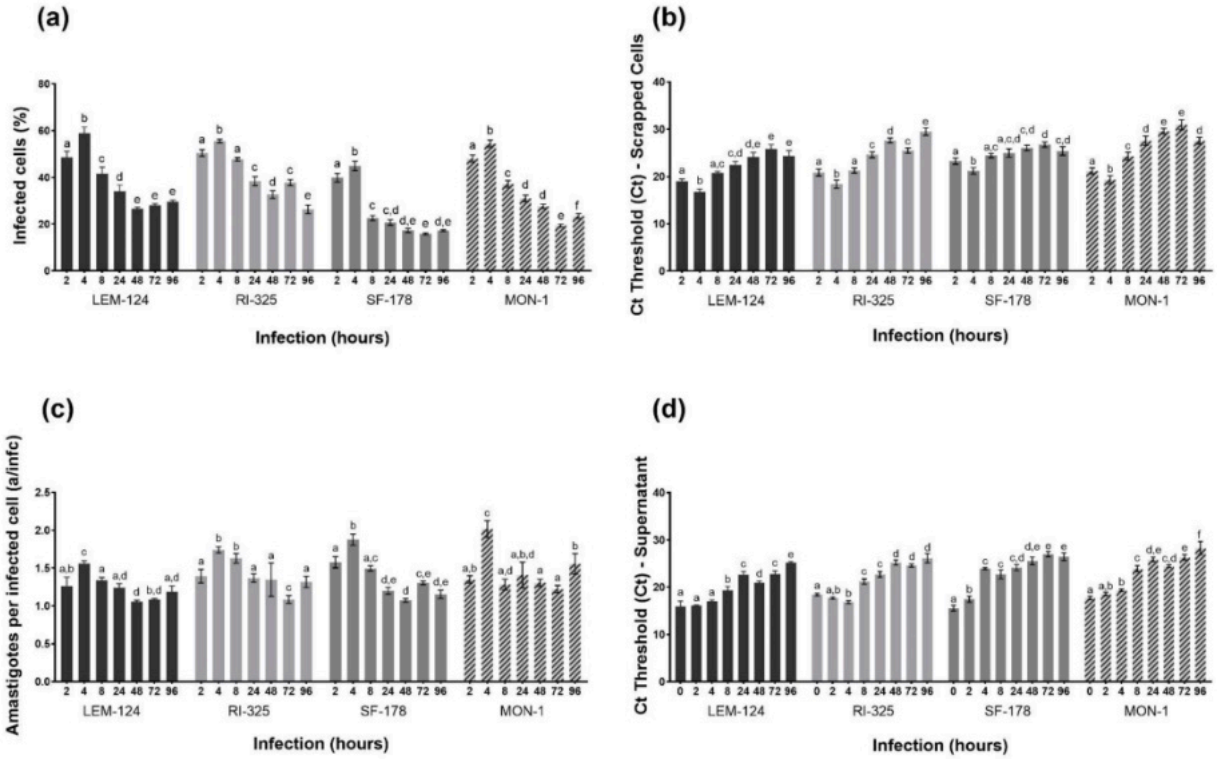

Fig. 2. *Leishmania* strains parameters evaluated by the percentual of infected cells under optical microscopy (a); scrapped infected cells DNA amplification expressed as Threshold Cycle (b); average number of amastigotes in each infected cell (a/infc) (c); and supernatant DNA amplification expressed as Threshold Cycle (Ct) (d), according to the time points. Significative differences ($p<0.05$) between time points on each infection parameter for each strain are indicated by different letters; same letters indicate no significative difference.

CRediT authorship contribution statement

Viviane Noll Louzada-Flores: Investigation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. Maria Stefania Latrofa: Investigation, Supervision, Methodology, Formal analysis, Writing – review & editing. Maria Stella Lucente: Methodology, Writing – review & editing. Bibiana Paula Dambro ś: Validation, Writing – review & editing. Jairo Alfonso Mendoza-Roldan: Investigation, Writing – review & editing. Ilaria Varotto-Boccazzi:

Investigation, Writing – review & editing. Giulia Maria Cattaneo: Investigation, Writing – review & editing. Gerald F Spaṫh: Investigation, Writing – review & editing. Alessio Buonavoglia: Investigation, Writing – review & editing. Domenico Otranto: Investigation, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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7.1.5 Development of a novel ddPCR assay for the simultaneous detection of the protozoan parasites *Leishmania* infantum and *Leishmania tarentolae*

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Abstract

Background *Leishmania*ses, caused by protozoan parasites of the genus *Leishmania*, are vector-borne diseases occurring mainly in the tropics and subtropics of the world, as well as in the Mediterranean Basin. In this area, the mammalian pathogen *Leishmania infantum* is endemic, along with the reptile-associated *Leishmania tarentolae*. The two species occur in sympatry, and there is evidence that the exposure to *L. tarentolae* in mammalian hosts may elicit a protective immune response towards pathogenic *Leishmania* species. Accurate detection methods for both species are therefore crucial for gathering comprehensive information on the epidemiology of *Leishmania*ses. In microbiological diagnosis, limits in detection performance imply the risk of false negatives and other issues, which highlights the need for sensitive methods.

Methods Here, we developed a droplet digital polymerase chain reaction assay targeting the kinetoplast minicircle DNA, for the simultaneous and differential detection of *L. infantum* and L. *tarentolae*. The assay features primers designed to bind to both species and species-specific probes. The assay was validated on three cultured isolates for each species, whose cells were

spiked into *Leishmania*-negative dog blood, and on *Leishmania*-positive sand flies. Sensitivity was assessed with testing serial dilutions, and specificity was evaluated by assessing the cross-reactivity of the probes with the controls of *Leishmania*-free dog blood and male sand fly DNA.

Results The assay demonstrated high sensitivity, with a limit of detection corresponding to one *Leishmania* cell in the reaction mix for isolates of both *L. infantum* and L. *tarentolae*. Limited cross-reaction of the L. *tarentolae*-targeting probe was observed on *L. infantum* isolates. No cross-reaction was observed with the controls of *Leishmania*-free dog blood and male sand flies.

Conclusions The protocol can represent a valuable method for comprehensive surveillance in both canine hosts and sand flies in areas in which *L. infantum* and *L. tarentolae* occur in sympatry.

Keyword Droplet digital PCR, *Leishmania* infantum, *Leishmania tarentolae*, leishmaniasis, Epidemiology, Sand flies

Background

*Leishmania*ses are vector-borne diseases caused by protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), which are transmitted to their vertebrate hosts via the bite of infected female sand flies (Diptera: Psychodidae: Phlebotominae) [1]. These diseases represent a significant public health burden in various parts of the world, particularly in tropical and subtropical regions, and are also endemic in the Mediterranean Basin [2]. In Italy, human cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL), as well as canine leishmaniasis (CanL), are endemic and are caused by *Leishmania infantum* Nicolle, 1908 [3]. In the country, the main vectors of *L. infantum* are sand flies of the species *Phlebotomus perniciosus* Newstead, 1911 and *Phlebotomus perfiliewi* Parrot, 1930 [4, 5]. In addition, the reptile-associated *Leishmania tarentolae* Wenyon, 1921 has also been detected in Italy [5–9]. This parasite is regarded as non-pathogenic to humans and mammals in general, and is therefore exploited as an antigen production and vaccine development platform [10, 11]. The ecology and epidemiology of L. *tarentolae*, however, remain poorly understood. *Leishmania tarentolae* is vectored by sand flies of the species *Sergentomyia minuta* (Rondani, 1843), which feed mainly on reptiles [12]. Nevertheless, this parasite may circulate in vectors

other than *S. minuta*, as *L. tarentolae* DNA has been detected in the sand fly *P. perniciosus* in Italy [6, 13]. This finding is in accordance with the development of *L. tarentolae* in *P. perniciosus* under laboratory conditions [14]. The parasite may also circulate in non-reptilian hosts, and evidence of this was gathered in central and southern Italy by molecular detection of *L. tarentolae* in dogs and humans [9, 13]. Moreover, *S. minuta* sand flies have been reported to feed on humans both in captivity [15] and in the wild [16, 17], and may therefore transmit *L. tarentolae* to non-reptilian hosts. This is particularly interesting given that exposure of mammalian hosts to *L. tarentolae* may elicit a protective immune response towards pathogenic *Leishmania* species [18]. On the other hand, in Italy, the DNA of *L. infantum* has been detected in sand flies of the species *S. minuta* [13, 19]. Furthermore, the DNA of both species of *Leishmania* has been detected simultaneously in sand fly vectors and vertebrate hosts in the country [8, 9]. In addition, in Italy, *S. minuta* and *P. perniciosus* have repeatedly been collected in the same sites, raising the potential occurrence of *L. infantum* and *L. tarentolae* in (i) sympatric conditions, (ii) unconventional vectors (e.g. *L. infantum* in *S. minuta* and *L. tarentolae* in *Phlebotomus* spp.), and iii) unconventional hosts (e.g. *L. infantum* in reptiles and *L. tarentolae* in mammals) [6, 9]. Therefore, accurately detecting both *L. infantum* and *L. tarentolae* in sand flies and vertebrate hosts is essential for reconstructing leishmaniasis epidemiological scenarios and for providing insights into the transmission dynamics of the infection. Traditional diagnostic methods, such as microscopy and polymerase chain reaction (PCR), may have limitations in terms of sensitivity, specificity, and quantification accuracy, particularly when dealing with complex biological samples containing low concentrations of parasite DNA [20]. For this reason, droplet digital PCR (ddPCR) has emerged as a powerful tool for the detection and quantification of nucleic acids with unparalleled sensitivity, specificity, and accuracy. By partitioning PCR reactions into thousands of individual droplets, ddPCR enables absolute quantification of target DNA molecules, overcoming many of the limitations associated with traditional PCR methods [20]. For this reason, ddPCR has been employed in the detection of a variety of pathogenic microorganisms [21–25]. Only three ddPCR protocols for the detection of *Leishmania* DNA have been published to date: the first protocol was developed for the detection of *Leishmania* braziliensis Vianna, 1911 DNA in samples obtained from patients affected by cutaneous leishmaniasis [26], the second for the quantification of *L. infantum* in dogs [27], and the third protocol was validated on six *Leishmania* isolates representing five species [28]. In addition, to the best of our knowledge, no ddPCR protocols

have been developed and published for the screening of *Leishmania* spp. in sand fly vectors. We developed a novel ddPCR assay targeting the minicircles of the kinetoplast DNA (kDNA) for the sensitive and specific simultaneous detection of *L. tarentolae* and *L. infantum* and validated this method on DNA samples derived from laboratory-maintained *Leishmania* strains, spiked to *Leishmania*-free dog blood, and from wild-caught sand flies. The method showed high sensitivity, allowing for the detection of low parasite loads, and high specificity, showing only minimal cross-reactivity.

Methods

Oligonucleotide design

A region encompassing the Conserved Sequence Blocks 1 and 2 (CSB 1 and CSB 2) of the kinetoplast minicircles was chosen as the ddPCR target. Sequences from *L. infantum*, L. *tarentolae*, L. *donovani* (Laveran et Mesnil, 1903), L. major Yakimoff et Schokhor, 1914, L. tropica Wright, 1903, and L. braziliensis Vianna, 1911 were aligned to design the primers and the probes. The primers were designed to amplify both *L. tarentolae* and *L. infantum* DNA, whereas the probes were designed to be species-specific. The resulting amplicon was 56 bp long. The absence of cross-reaction and formation of secondary structure of primers and probes was verified using the PrimerPooler software [29]. The probes were labelled with the FAM fluorophore for *L. tarentolae* and with the HEX fluorophore for *L. infantum*. All the primers and probes were manufactured by Eurofins Genomics (Konstanz, Germany). The oligonucleotide sequences are reported in Table 1.

ddPCR experiment conditions

Each ddPCR reaction targeting *L. tarentolae* and *L. infantum* minicircles included 11 µL of Bio-Rad ddPCR Supermix for Probes (No dUTP) (Bio-Rad, Hercules, CA, USA) primers at a final concentration of 900 nM and probes at a final concentration of 250 nM, 0.78 µL of Milli-Q water, and 5 µL of DNA. The assays were performed in the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA). The PCR reaction was conducted on a SimpliAmp Thermal Cycler (Applied Biosystems, Waltham, MA, USA) following a thermal protocol of 10 min at 95 °C for denaturation, 50 cycles of annealing at 50 °C, and elongation at 72 °C. Annealing temperatures were chosen after gradient PCR experiments (see below).

Dog blood handling

The methods to handle dog blood, which is derived from a previous study [30], were approved by the Clinvet Institutional Animal Care and Use Committee (no. CG1331CVMO22/216). All experiments involving the use of dog blood were performed in accordance with the ARRIVE guidelines and international regulations. All methods were carried out in accordance with relevant guidelines and regulations.

ddPCR assay validation

***Leishmania*-cultured cells spiked in dog blood**

The ddPCR assay validation was performed on DNA extracted from both *L. tarentolae* and *L. infantum* cultures spiked into *Leishmania*-negative dog blood, derived from another study [30]. For L. *tarentolae*, the commercial P10 strain (Jena Bioscience, Jena, Germany), maintained in Brain Heart Infusion (BHI) broth, was used. Alongside the P10 strain, the DNA of wild *L. tarentolae* strains "W", isolated from the sand fly vector S. minuta, and "RI325", isolated from the reptile host Tarentola mauritanica (Linnaeus, 1758), were used. Both wild strains were isolated at the Parasitology and Micology laboratory of the University of Bari Aldo Moro and were maintained in Schneider broth. For *L. infantum*, the DNA of the MHOM/TN/80/IPT-1 reference strain (Office International des Epizooties [OIE] Reference Laboratory National Reference Center for leishmaniasisC.Re.Na.L., Palermo, Italy) and the "S" and "G" strains recently isolated from dogs from southern Italy were used. The dog strains were isolated at the University of Bari Aldo Moro and maintained in Schneider's Drosophila medium.

Leishmania cells were counted in a counting chamber under a Leica DM300 optical microscope (Leica Microsystems, Germany) and then spiked to 100 µl of *Leishmania*-negative dog blood. Amounts of 10^6, 5×10^5, 10^5, 5×10^4, 10^4, 5×10^3, 10^3, 5×10^2, 10^2, 50, and 20 cells were used. These quantities correspond to 10^4, 5×10^3, 10^3, 5×10^2, 10^2, 50, 10, 5, 1, 0.5, and 0.2 cells/µl of blood, respectively. The mixture was then subjected to DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The elution step was carried out in 100 µl, and 5 µl of DNA was added to the ddPCR mix. This means that the estimated *Leishmania* DNA present in the mix should correspond to 1/20 of the DNA of the original cell quantities, namely to 5×10^4, 25×10^3, 5×10^3, 25×10^2, 500, 250, 50, 25, 5, 2.5, and 1 cell, respectively.

The annealing temperatures of the assay thermal protocol were selected based on gradient PCR experiments, with temperatures ranging from 45 °C to 60 °C. Each of the isolated serial dilutions was run in triplicate.

Cross-reactivity testing

The potential of cross-reactivity of the probes was assessed by testing the DNA extracted from the dog blood which *Leishmania* cells where spiked to, and the DNA extracted from four male sand flies, collected during routine monitoring activity carried out by laboratory members. Wild-caught sand flies Fifteen female sand fly DNA samples from the collection of the Parasitology and Micology laboratory of the University of Bari Aldo Moro were used to validate the ddPCR assay (n. 13 = S. minuta; n. 2 = P. *perniciosus*). These sand flies have been collected in the Valenzano municipality, Bari district. Their DNA samples have been previously screened for both *L. tarentolae* and *L. infantum* with a duplex quantitative real-time PCR (qPCR) assay targeting the first Internal Transcribed Spacer (ITS1) region [31].

Statistical analyses

All the statistical analyses of the study were carried out in the R programming environment (https://www.r-proje ct.org/).

Oligonucleotide name	Type	Target gene	Species	Sequence (5′-3′)
MC_Leish_F	Forward primer	Minicircles CSB 1 and CSB 2	*L. tarentolae* + *L. infantum*	TCCCAAACTTTTTAGGTC
MC_Leish_R	Reverse primer	Minicircles CSB 1 and CSB 2	*L. tarentolae* + *L. infantum*	GCRWTTTTGGCCAWTTTTTG
MC_tar_FAM	Probe FAM	Minicircles CSB 1 and CSB 2	*L. tarentolae*	CCGAAAACCGAAAAATGCATGCA
MC_inf_HEX	Probe HEX	Minicircles CSB 1 and CSB 2	*L. infantum*	GCGAAAACSGAAAAATGGGTGCA

Table 1 Oligonucleotides designed and employed for the validation of the ddPCR assay

Results

ddPCR annealing set-up

The optimal annealing temperature for detecting *L. tarentolae* and *L. infantum* was determined through experimentally established gradients using *Leishmania*free dog blood spiked with *Leishmania* cells coming from the six above-mentioned laboratory-maintained strains (see Methods section). For L. *tarentolae*, the best amplification performance was obtained at 55 °C. A non-specific population of droplets was present at 3500 Relative Fluorescence Units (RFUs) amplitude but was clearly distinguishable from the droplets of interest, which form at around

5500–6000 RFUs amplitude (Fig. 1). “Rain” was observed between the non-specific population and the droplets of interest.

As for *L. infantum* detection, the best amplification performance was obtained at 50 °C instead of 55 °C. The maximum density of droplets in positive samples was localized between 3000 and 4000 RFUs, with more “rain” observed compared to the *L. tarentolae* isolates. However, the positive droplets were distinguishable from the negative droplets occurring at around 2000 RFUs. A statistically significant difference was observed after comparing the concentrations of target DNA obtained from the above-mentioned serial dilutions of *L. infantum* MHOM/TN/80/IPT-1 reference strain at 50 °C and 55 °C of annealing (Shapiro–Wilk test for normality P-value = 0.004754; Wilcoxon signed rank exact test, P-value = 0.01563). In summary, the annealing temperature of 55 °C resulted to be too high for *L. infantum*, and a temperature of 50 °C was chosen instead (Fig. 2). When testing *L. tarentolae* samples at 50 °C annealing, no significant differences were observed with regard to the results obtained at 55 °C, considering the above-mentioned serial dilutions of the *L. tarentolae* P10 isolate (ShapiroWilk test for normality P-value = 0.006; Wilcoxon signed rank exact test, P-value = 0.1563). The only difference was observed in the droplets’ amplitude: at 50 °C annealing, positive droplets showed an amplitude of ~ 8000 RFUs, while the non-specific population had an amplitude of 5000 RFUs. The positive and negative droplets remained clearly separated. Rain was still observed. In conclusion, for duplex application targeting both *Leishmania* species, we decided to set the annealing temperature at 50 °C.

Assay sensitivity and specificity

The presented ddPCR assay showed high sensitivity. The assay limit of detection for both species was 0.2 *Leishmania* cells/µl of dog blood. In other words, the assay is able to detect the DNA of one *Leishmania* cell present in the ddPCR mix (0.2 cells/5 µl of DNA in the mix).

During the assay validation process, specificity was assessed by testing the *Leishmania*-spiked dog blood, inverting the probes (i.e. FAM included in the mix for *L. infantum* and HEX for L. *tarentolae*). No crossreaction occurred for the HEX probe with regards to L. *tarentolae*-spiked blood, while one to four FAM-positive droplets were observed in *L. infantum*-spiked blood, regardless of the parasite concentrations (Fig. 3).

The ddPCR assay also showed high specificity: the HEX probe, targeting *L. infantum*, showed no crossreactivity with *Leishmania*-free dog blood. Regarding the FAM probe, targeting L.

tarentolae, only a single droplet resulted positive at 50 °C of annealing while testing dog DNA (Fig. 4). While testing *Leishmania*-free male sand flies (see Methods section), the HEX probe targeting *L. infantum* did not hybridize with the DNA extracted from the insects. Considering the FAM probe, two positive droplets were detected in three out of four male DNA samples, while in the fourth one positive droplet was detected (Fig. 4). However, while testing DNA of female *L. infantum* positive sand flies (see next section) no cross reaction occurred between the FAM probe and the DNA of those samples.

The concentration, expressed as copies of target/µL, of the serial dilutions of the *L. tarentolae* "W" isolates and all *L. infantum* isolates did not exhibit a constant decrease between the samples of 1, 0.5, and 0.2 parasite cells/µL of dog blood. This may be due to the effect of chance or to dilution errors that can occur at such low cell concentrations.

ddPCR vs ITS-1 qPCR

The ddPCR, targeted to the minicircle, and the qPCR, targeted to the ITS-1 (see Methods section), showed congruence in their results on 11 out of 15 sand fly DNA samples (73.3%). Two *S. minuta* sand flies resulted positive to *L. tarentolae* only at qPCR testing, but positive to both *L. tarentolae and L. infantum* after the application of the ddPCR herein described. In addition, one *S. minuta* sand fly and one *P. perniciosus* sand fly showed positivity to *L. tarentolae* after qPCR, but ddPCR resulted positive to *L. infantum*, and negative to L. *tarentolae*. The ddPCR target amplicon concentrations and qPCR quantification cycles (cqs) are reported in Table 2. The ddPCR results are reported in Fig. 5.

Estimation of parasite number in sand flies

Comparing the concentrations obtained from ddPCR quantification of serial *Leishmania* cell dilutions in dog blood can provide a broad estimate of the number of parasite cells infecting the tested sand flies. Three individual sand fly females showed the maximum ddPCR concentration of 1,000,000 copies of amplified target DNA/µl. All the other samples ranged from less than one copy of target/µl to ~ 11,500 copies of target/µl. Based on these results, we estimated parasite numbers, which ranged from less than one to 5000 or more, in positive sand flies, as reported in Table 3. We emphasize that, in saturated samples (i.e. those with maximum ddPCR concentration of 100,000 copies), the number of parasite cells at 5000 could be an underestimation.

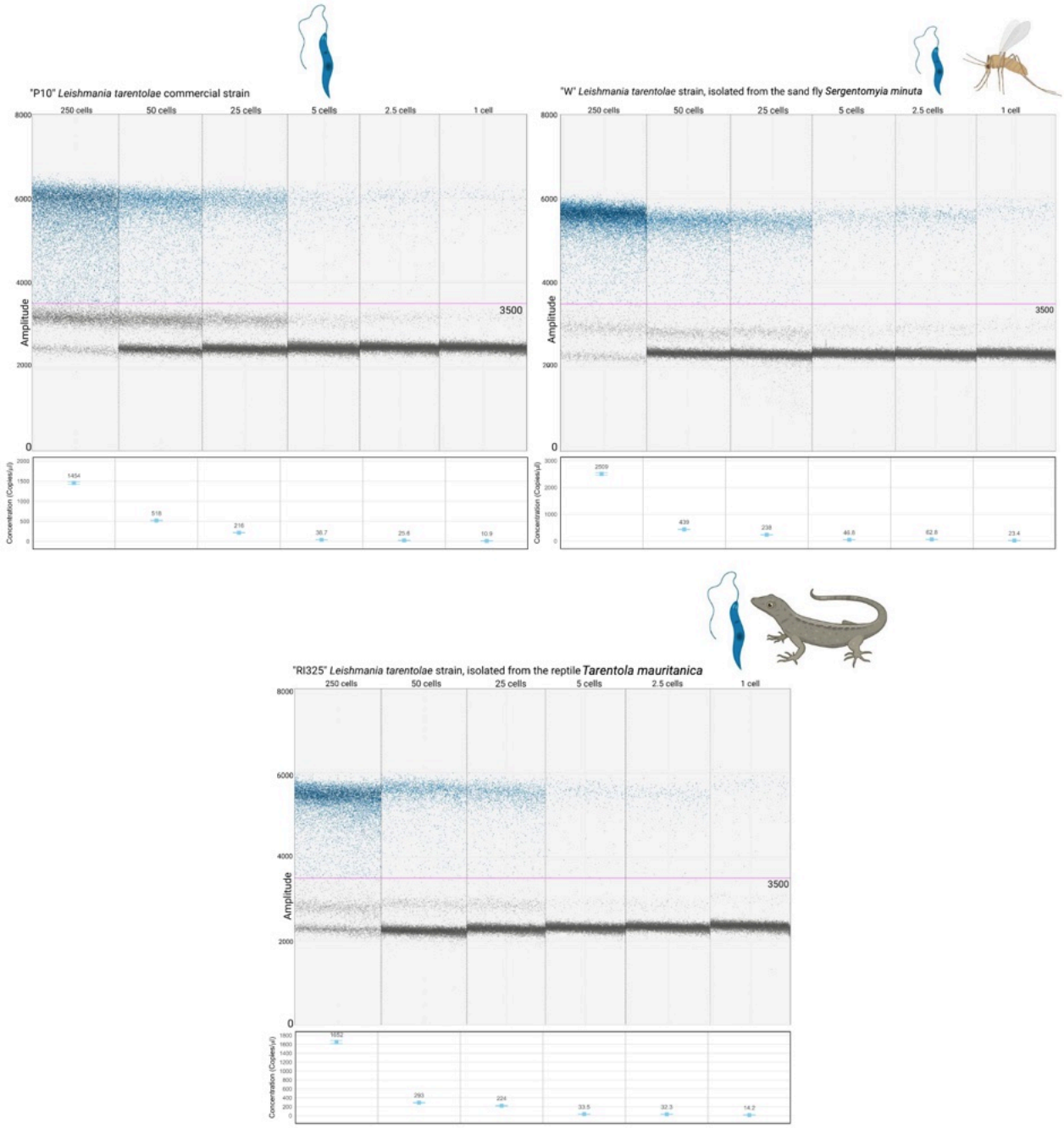

Fig. 1 ddPCR assay sensitivity assessment on dog blood spiked with serial dilutions of the commercial P10 *L. tarentolae* strain (Jena Bioscience), and the wild "W" and "RI325" *L. tarentolae* strains. Annealing temperature of 55 °C. The limit of detection is 0.2 *Leishmania* cells/µl of DNA extracted from dog blood added to the ddPCR mix, corresponding to one *Leishmania* cell present in the ddPCR mix. The fluorescence amplitude intensity is indicated on the y-axis. Each dot represents an individual droplet. The pink line represents the amplitude threshold above which a droplet is assigned as positive (i.e. it contains one or more target copies, coloured in blue), and below which a droplet is assigned as negative (i.e. it contains no target copies, coloured in grey). For each sample, the number of *Leishmania* cells per µL of

input DNA sample is shown above, while the concentration of target DNA copies per µL of input DNA sample is displayed in the box below

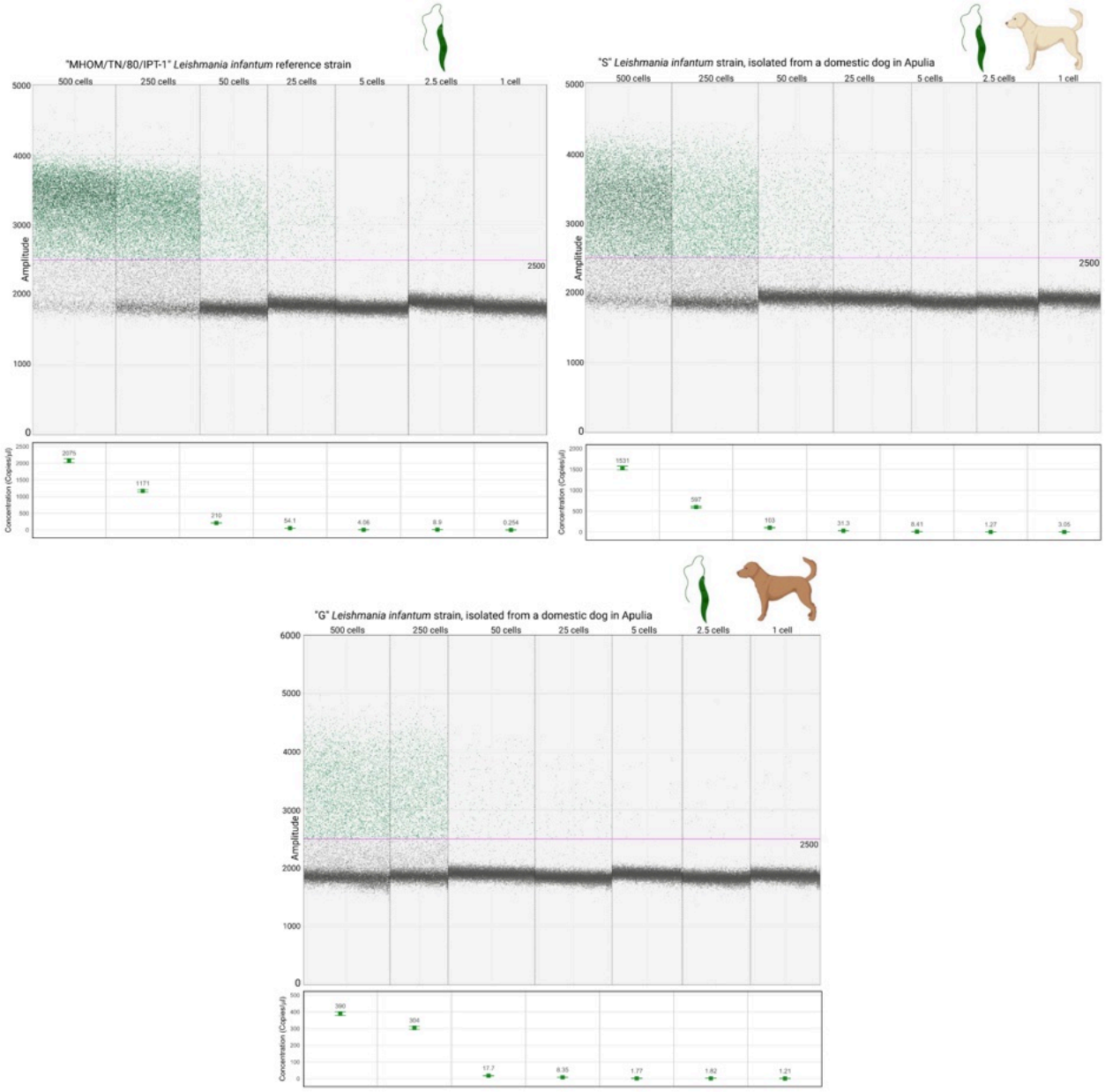

Fig. 2 ddPCR assay sensitivity assessment on dog blood spiked with serial dilutions of the *L. infantum* "MHOM/TN/80/IPT-1" reference strain (OIE Reference Laboratory National Reference Center for leishmaniasis—C.Re.Na.L., Palermo, Italy), and the wild "S" and "G" *L. infantum* strains (University of Bari Aldo Moro). Annealing temperature of 50 °C. The limit of detection is 0.2 *Leishmania* cells/µl of DNA extracted from dog blood added to the ddPCR mix, corresponding to one *Leishmania* cell present in the ddPCR mix. The fluorescence amplitude intensity is indicated on the y-axis. Each dot represents an individual droplet. The pink line represents the amplitude threshold above which a droplet is assigned as positive (i.e. it contains one or more target copies, coloured in green), and below which a droplet is assigned as negative (i.e. it contains no target copies, coloured in grey). For each sample, the number of *Leishmania*

cells per µL of input DNA sample is shown above, while the concentration of target DNA copies per µL of input DNA sample is displayed in the box below

Discussion

The ddPCR assay described in the present research demonstrated high sensitivity and specificity. Setting the annealing temperature to 50 °C does not represent a limit for the detection of L. *tarentolae*, as the non-specific population of droplets is clearly distinguishable from the positive droplets of interest. The limit of detection for *L. tarentolae* corresponds to 0.2 *Leishmania* cells/µl of dog blood, or, in other words, to one *Leishmania* cell included in the reaction mix, therefore, the assay can represent a powerful tool for estimating parasite prevalence in extensive epidemiological screenings.

Rain was observed in all samples. As our aim was to develop an assay for large epidemiological screenings, we kept a "conservative" threshold in terms of the amplitude of droplets that have to be considered positive. Excluding the rain by raising the threshold for positive droplets lowers the concentration detected by the assay. However, even by setting the threshold in a way in which all rain is excluded, the limit of detection of the assay does not change.

Our assay was characterized by minimal cross-reactivity of the probes with the non-target *Leishmania* species and with the controls represented by dog and sand fly DNA. The complete absence of cross-hybridization with the non-targets was observed in every experimental condition for the HEX probe targeting *L. infantum*. Limited hybridization of the FAM probe, targeted on L. *tarentolae*, occurred with non-targets instead. Indeed, up to four positive droplets were obtained analysing *L. infantum* samples and the controls of *Leishmania*-free dog blood and male sand flies. Therefore, in our assay, a result with fewer than five FAM-positive droplets and zero HEXpositive droplets should be classified as negative.

Of the 15 female *Leishmania*-positive sand fly samples tested with the ddPCR assay, 11 were consistent with the qPCR results obtained previously, using ITS-1 as the target [31]. Two *S. minuta* sand flies resulted positive only to *L. tarentolae* after qPCR, but also resulted positive to *L. infantum* after ddPCR, with a post-amplification target DNA concentration of less than one copy/µl. This result suggests a possible contact of these sand flies with *L. infantum* parasites, with the corresponding molecular signature undetected after the ITS-1-targeted qPCR. The ddPCR assay, instead, successfully revealed this signature, likely due to the use of kDNA minicircles as the target of the assay. These mitochondrial elements occur in thousands of copies per parasite [32], in contrast to the ITS-1 nuclear region, which is present in only 20 to

200 copies per cell [33]. This marked difference in copy number translates into a higher sensitivity of ddPCR, particularly when dealing with samples harbouring low parasite loads. The simultaneous detection of *L. tarentolae* and *L. infantum* in the tested sand flies is also in accordance with the finding of co-infected sand flies coming from the same area where the samples were collected [7]. One *S. minuta* and one *P. perniciosus* sand fly tested positive for *L. tarentolae* after ITS-1-targeted qPCR; however, after ddPCR application, the same samples were positive for *L. infantum* and negative for L. *tarentolae*. This incongruence may reflect mitonuclear discordance occurring in the *Leishmania* parasites infecting these sand flies. In mitonuclear discordance, nuclear and mitochondrial genomes have discordant evolutionary histories; this causes topological discordance between nuclear gene and mitochondrial gene-based phylogenies, ultimately resulting in incorrect species identification [34]. Mitonuclear discordance is the result of genetic exchange between different species, resulting in the formation of hybrids. The biological mechanism on the basis of mitonuclear discordance in *Leishmania* remains poorly understood, but there is experimental evidence that the genetic exchange between the parental species happens in the sand fly vector [34]. Therefore, we could interpret the discordance in the results we obtained as an example of mitonuclear discordance, occurring in two out of the 15 sand flies constituting the dataset tested in our study (frequency = 13.3%), with nuclear DNA (ITS-1) of *L. tarentolae* and mitochondrial DNA (kDNA) of *L. infantum*. A study in Peru described a similar frequency of New World *Leishmania* hybrids in samples obtained from CL human patients [35]. Our study provides the first evidence to date of mitonuclear discordance and possible hybridization between the representatives of two subgenera of the *Leishmania* genus, namely L. (*Leishmania*) and L. (*Sauroleishmania*). This also represents the first possible report of the phenomenon observed in wild-caught sand flies. The sympatric occurrence of *L. infantum* and *L. tarentolae* may enhance the possibilities of hybridization of these two *Leishmania* species [12]. This may have happened in the collection area of the sand flies tested in our study, in which the two *Leishmania* species coexist. Therefore, the use of both mitochondrial- and nuclear-targeted detection methods is recommended in areas where more than one species is present. The method of ddPCR does not require the construction of a standard curve because it relies on the absolute quantification of the target molecules. Nevertheless, serial dilutions can be useful to experimentally determine the assay limit of detection. By comparing the ddPCR quantification of the serial dilution of *Leishmania* cells in dog blood, we can perform a broad estimation of the parasite

cell number infecting the tested sand flies. The value of the concentration of *L. tarentolae* in three sand fly samples reached saturation (i.e. 1,000,000 copies of target/µl). This result is in accordance with the ITS-1 targeted qPCR cqs of these samples, which are amongst the lowest in the dataset. While preparing serial dilutions of *L. tarentolae* isolates employed in the ddPCR validation, the saturation was reached starting from 100,000 *L. tarentolae* cells (corresponding to 5000 parasite cells added in the reaction mix; not shown) spiked in dog blood. Therefore, the sand flies representing the abovementioned samples could have harboured 5000 or more *Leishmania* cells. One sand fly sample showed a concentration higher than 10,000 *L. tarentolae* cells (corresponding to 500 parasite cells added in the reaction mix; not shown). The number of parasites estimated for the other samples ranged from less than 1 to 250 parasites. The estimated parasite count of less than one cell in some samples may result from mere contact between the sand fly and parasite DNA, rather than indicating an established infection. Indeed, these samples showed higher cqs when tested with the ITS-1 targeted qPCR. Overall, the estimation of *Leishmania* cells in sand flies, although being approximate, is consistent with the quantifications reported in the literature [36–38]. However, our ddPCR assay suffers from some limitations, some of them intrinsic to the method. The ddPCR is more expensive than the qPCR. For instance, in qPCR the cost is approximately $1.70 per sample, while in ddPCR the cost for a single sample is ~$5.00. Moreover, ddPCR may be more prone to contamination than qPCR. Considering that ddPCR can detect minimal amounts of target DNA, the higher risk of contamination compared to qPCR is a disadvantage in the studies of *Leishmania* prevalence in sand flies. These insects can be collected and stored with methods by which they can come in close contact (i.e. sticky traps), and contamination may occur. Therefore, the assay may ultimately result in lower specificity due to a higher number of false-positive samples. To overcome this problem, as the limit of detection is as low, an experimental threshold should be set in order to identify possible cross-reactions, especially with other sand fly and *Leishmania* species. In fact, in this study, we were able to validate the assay only on *P. perniciosus* and *S. minuta* sand flies, and we did not include other *Leishmania* species apart from *L. tarentolae* and *L. infantum*.

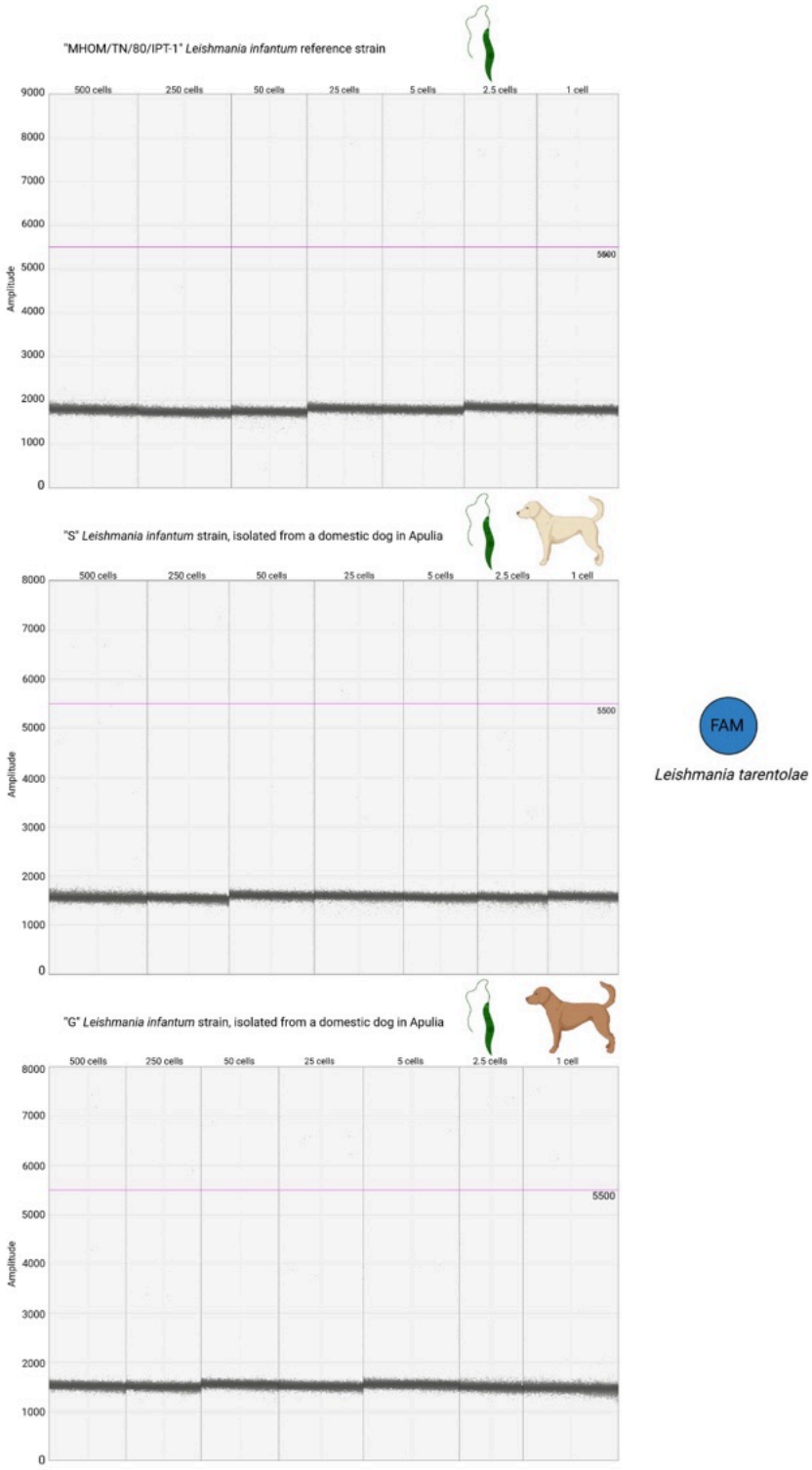

Fig. 3 ddPCR specificity assessment by testing for cross-reaction between the FAM probe and the *L. infantum* DNA extracted from the three isolates used in the study. Annealing temperature of 50 °C. A maximum of four FAM-positive droplets were detected when analysing *L. infantum* samples. Therefore, any result with fewer than five FAM-positive droplets should be considered negative. The fluorescence amplitude intensity is indicated on the y-axis. Each dot represents an individual droplet. The pink line represents the amplitude threshold above which a droplet

is assigned as positive (i.e. it contains one or more target copies, coloured in blue), and below which a droplet is assigned as negative (i.e. it contains no target copies, coloured in grey)

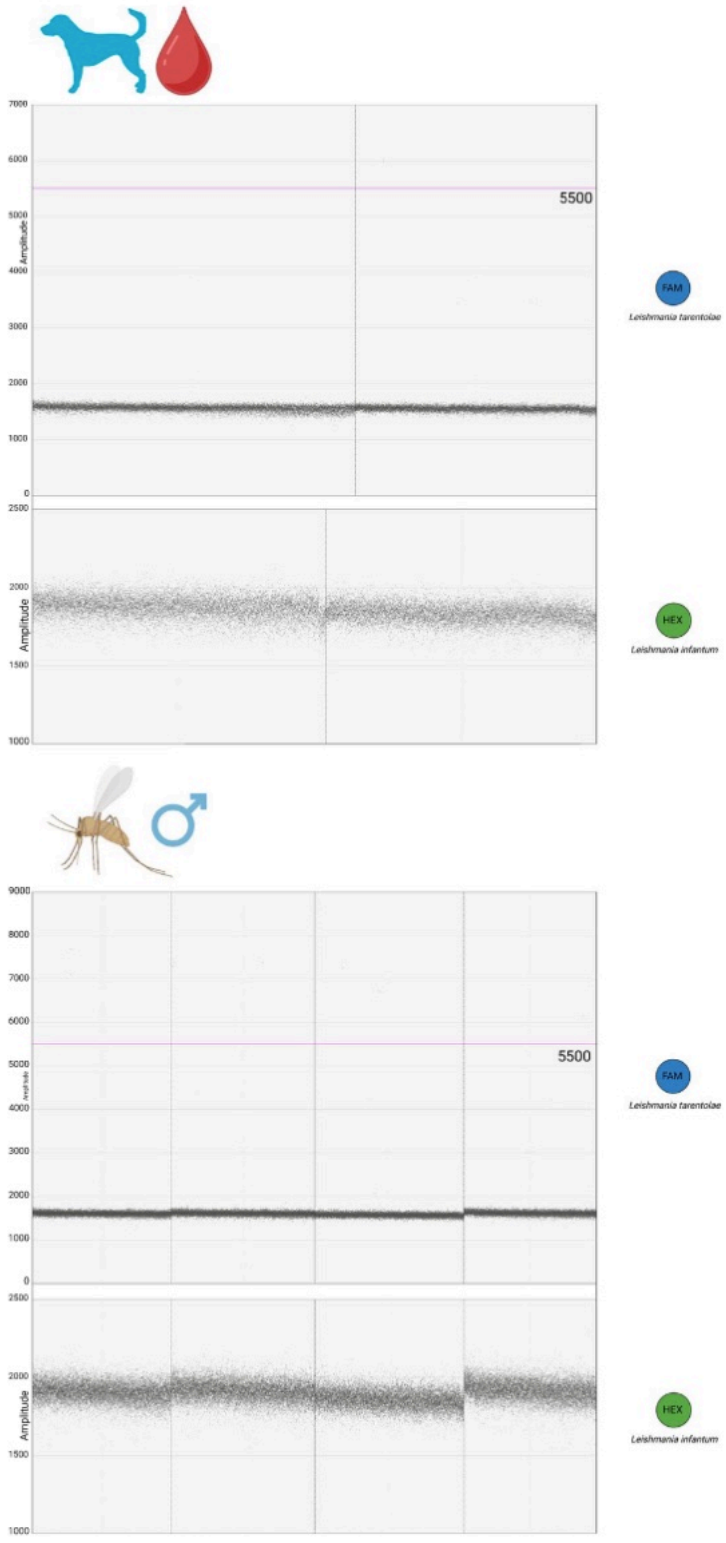

Fig. 4 ddPCR assay specificity assessment on the controls represented by DNA extracted from *Leishmania*-negative dog blood and male sand flies. Annealing temperature of 50 °C. Each sample has been tested with both the FAM probe, targeting L. *tarentolae*, and with the HEX probe, targeting L. infantum. A maximum of two FAM-positive droplets have been observed in the sand fly samples, whereas no droplets hybridized with the HEX probe. Given the threshold set after testing for cross-reaction between the FAM probe and the *L. infantum* DNA, the control samples should be considered negative. For each sample, the FAM result is shown in the box on the top, while the HEX result is displayed in the box below. The fluorescence amplitude intensity is indicated on the y-axis. Each dot represents an individual droplet. The pink line represents the amplitude threshold above which a droplet is assigned as positive (i.e. it contains one or more target copies, coloured in blue), and below which a droplet is assigned as negative (i.e. it contains no target copies, coloured in grey)

		cqs of ITS1-duplex qPCR (Latrofa et al., 2021)		ddPCR Concentration (copies of target/µl)	
Sand fly ID	**Species**	***L. tarentolae***	***L. infantum***	***L. tarentolae***	***L. infantum***
31	*S. minuta*	**24.9**	neg	**1000000 (max)**	0
74	*S. minuta*	**37.51**	neg	**5.74**	0
190	*S. minuta*	**36.19**	neg	**5.19**	0
192	*S. minuta*	**23.84**	neg	**1,000,000 (max)**	0
197	*S. minuta*	**32.4**	neg	**146**	0
199	*S. minuta*	**30.38**	neg	**100**	0
202	*S. minuta*	**30.32**	neg	**214**	0
279	*S. minuta*	**22.4**	neg	**1,000,000 (max)**	0
288	*S. minuta*	**19.63**	neg	**11415**	0
454	*S. minuta*	**34.65**	neg	**31.4**	0
364	*P. perniciosus*	neg	**29.69**	0	**291**
292	*S. minuta*	**30.36**	neg	**2169**	**0.759**
372	*S. minuta*	**33.46**	neg	**0.858**	**0.132**
365	*S. minuta*	**35.03**	neg	0	**1.85**
393	*P. perniciosus*	**36.54**	neg	0	**1.31**

Table 2 Results of ITS-1-targeted qPCR and ddPCR on the same female *Leishmania*-positive samples.

Samples whose corresponding cells are highlighted in red showed positivity to both species after ddPCR; samples whose corresponding cells are highlighted in blue were positive only for *L. infantum* after ddPCR, despite having shown positivity for *L. tarentolae* in the qPCR results

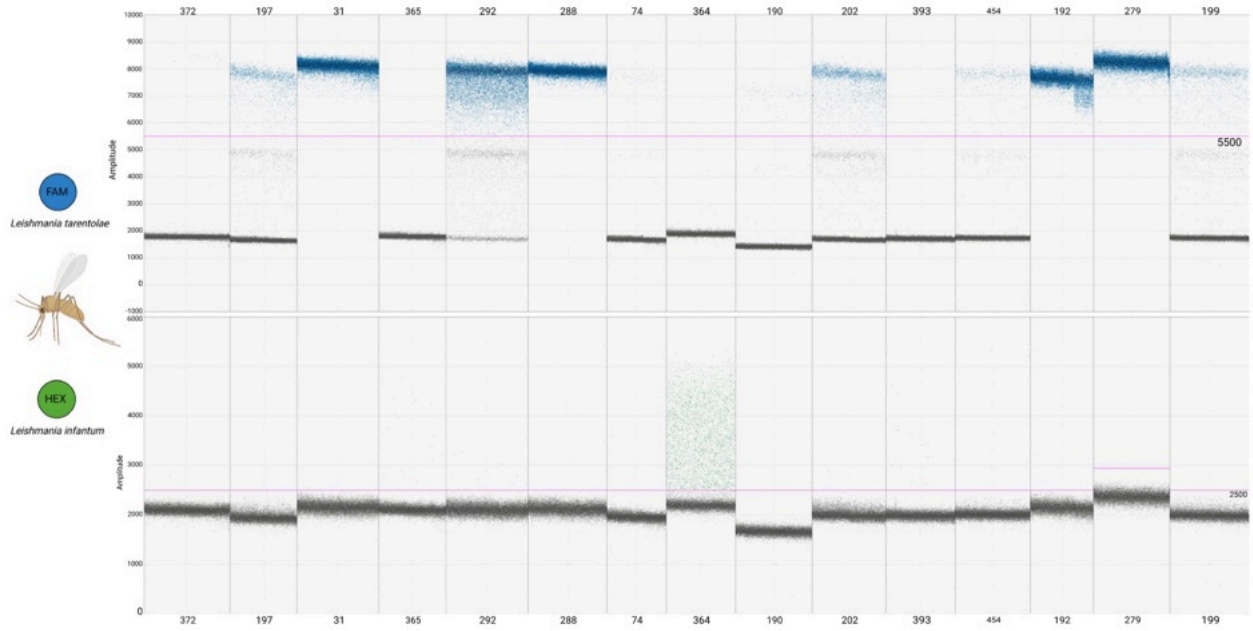

Fig. 5 Results of the ddPCR testing of the qPCR-*Leishmania*-positive sand flies from the collection of the University of Bari Aldo Moro. Annealing temperature of 50 °C. Each sample has been tested with both the FAM probe, targeting L. *tarentolae*, and with the HEX probe, targeting L. infantum. For each sample, the FAM result is shown in the box on the top, while the HEX result is displayed in the box below. Sample names are indicated at the bottom of the HEX box. The fluorescence amplitude intensity is indicated on the y-axis. Each dot represents an individual droplet. The pink line represents the amplitude threshold above which a droplet is assigned as positive (i.e. it contains one or more target copies, coloured in blue or green), and below which a droplet is assigned as negative (i.e. it contains no target copies, coloured in grey)

Sand fly ID	Species	Estimated *L. tarentolae* cells	Estimated *L. infantum* cells
31	*S. minuta*	5000 and up	0
74	*S. minuta*	less than 1	0
190	*S. minuta*	less than 1	0
192	*S. minuta*	5000 and up	0
197	*S. minuta*	2.5—5 parasites	0
199	*S. minuta*	2.5—5 parasites	0
202	*S. minuta*	~25 parasites	0
279	*S. minuta*	5000 and up	0
288	*S. minuta*	Over 500	0
454	*S. minuta*	~1 parasite	0
364	*P. perniciosus*	0	50–250 parasites
292	*S. minuta*	~250 parasites	less than 1 parasite
372	*S. minuta*	less than 1 parasite	0
365	*S. minuta*	0	~1 parasite
393	*P. perniciosus*	0	less than 1 parasite

Table 3 Estimated number of parasite cells in relation to concentration values of "W" isolate for *L. tarentolae* and "G" isolate for L. infantum

Conclusions

In this study, we present the first ddPCR protocol developed for the simultaneous detection of *L. tarentolae* and *L. infantum* parasites in both reservoir hosts and vectors. The assay exhibited high sensitivity, detecting low levels of DNA of both *L. tarentolae* and *L. infantum* parasites spiked into dog blood samples. This demonstrates the assay's utility for reliable *Leishmania* detection in dog blood, underscoring its potential as a valuable tool for clinical diagnostics and early intervention in canine leishmaniasis. To our knowledge, this is the first ddPCR assay validated for use on sand flies. By enabling accurate detection of *Leishmania* in sand fly populations, this assay could significantly enhance monitoring efforts and inform targeted control strategies in endemic regions. This dual application makes the protocol highly valuable for comprehensive surveillance in both canine hosts and sand flies. To prevent misinterpretation of results, we highlight the importance of establishing an experimental threshold, particularly for L. *tarentolae*, to avoid false positives. Additionally, we recommend that screening for *Leishmania* parasites include both mitochondrial and nuclear targets to account for potential mitonuclear discordance.

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Author contributions

Conceptualization, S.E. and A.A.; Methodology, S.E., A.A., and G.M.C.; Formal analysis, A.A., S.E., and M.B.; Writing—original draft preparation, A.A.; Writingreview and editing, S.E., C.B., I.V.B., R.M., A.M., V.G., and M.S.C.; Visuals preparation; Supervision, J.M.R. and D.O.; Funding acquisition, S.E. and C.B.

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Availability of data and materials

All the data generated in the paper are available in the paper itself.

Declarations

Ethics approval and consent to participate

The dog blood used for the presented research was obtained from a previous study. [30]. The methods to handle dog blood were approved by the Clinvet Institutional Animal Care and Use Committee (n° CG1331-CVMO22/216).

Consent for publication

Not applicable.

Competing interests

S.E. is an Associate Editor for Parasites & Vectors, and A.M. is employed by VisMederi Srl. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationship.

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7.1.6 Sand Fly Fauna and Prevalence of *Leishmania* spp. in a Newly Investigated Area of Northern Italy: Emerging Epidemiological Scenarios?

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Abstract

Since the 1990s, cases of canine leishmaniasis (CanL) due to *Leishmania infantum* have risen in northern Italy due to dog translocation and movement as well as for climate-driven sand fly population growth. In this geographical region, for a long time regarded as non-endemic for CanL, *L. infantum* is generally transmitted by the sand flies of the genus Phlebotomus. Other sand flies, such as Sergentomyia minuta, have been less investigated, also because they were only considered as vectors of the nonpathogenic herpetophilic *Leishmania tarentolae*. Our study investigates sand fly species composition and *Leishmania* spp. prevalence in hilly areas of northern Italy, in the Bergamo district. Sand flies were sampled with both sticky and light traps. Common wall lizards Podarcis muralis have been captured in the same areas, for the collection of biological samples: blood, feces, tissues, and gut-associated secretions. Sand flies were identified morphologically (males) and molecularly (females). The collected samples were tested for the presence of *Leishmania* spp. via conventional and digital droplet PCR. Our study reports the presence of *Phlebotomus perniciosus*, *Phlebotomus* neglectus, and *S. minuta* in the investigated area. We report the first detection of L. *tarentolae*, a *Sauroleishmania* species, in northern Italy, in both sand fly vectors (S. minuta, P. *perniciosus*, and P. neglectus) and reptile hosts. Additionally, *L. infantum* DNA was detected, for the first time, in sand flies and reptiles in the district, spatially overlapping with previously reported local CanL cases. Our

study reported the presence of three sand fly species in the Bergamo district, a *Sauroleishmania* species (the first record in northern Italy), and the occurrence of *L. infantum*. We emphasize the importance of including both *S. minuta* sand flies and synanthropic reptiles in leishmaniasis screenings for a better understanding of the epidemiology of the disease.

Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are small hematophagous insects of medical and veterinary importance, as they are the vectors of many viral, bacterial, and parasitic agents, such as those of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), the etiological agents of *Leishmania*ses. These diseases pose a major public health concern in several regions worldwide, especially in the tropics and the subtropics, and are also endemic to the Mediterranean area [1]. In Italy, canine leishmaniasis (CanL) and the human cutaneous (CL) and visceral (VL) forms of the disease are caused by *Leishmania infantum* Nicolle, 1908 [2, 3]. In this country, sand flies of the species *Phlebotomus perniciosus* Newstead, 1911, and *Phlebotomus perfiliewi* Parrot, 1930, are considered the main vectors of *L. infantum* [4, 5]. *Phlebotomus* neglectus Tonnoir, 1921, and *Phlebotomus* ariasi Tonnoir, 1921, are also present in Italy, and have been recognized as vectors of *L. infantum* in other parts of Europe [6–9]. Since the 1990s, both human *Leishmania*ses and CanL, traditionally considered restricted to southern and central Italy, have incremented their diffusion and their overall prevalence and incidence [10–14]. Cases of both human CL and VL, as well as CanL cases, have then been reported in the northern regions of the country [14–17]. Indeed, autochthonous cases of canine and human *Leishmania* infections have been recorded from all the northern Italian regions [14, 15]. The above mentioned changes in the epidemiological scenarios have been attributed, among others, to the traveling or relocation of infected dogs coming from hyperendemic areas [17] and the expansion of sand fly populations into new territories, likely as a consequence of climatic and environmental changes [16]. In this context, the increased density of *P. perniciosus* and P. neglectus populations northward has been associated with the emergence of autochthonous *Leishmania*ses cases [18]. Other sand fly species, such as *P. perfiliewi*, P. ariasi, *Phlebotomus* mascitti Grassi, 1908, *Phlebotomus papatasi* (Scopoli, 1786), and *Sergentomyia minuta* (Rondani, 1843) have also been reported in northern Italy [13, 19–22]. The herpetophilic *S. minuta* is one of the most abundant and widespread species in the Mediterranean area, but it is normally not included in *Leishmania* spp. epidemiological studies,

as it is regarded as the vector of the reptile-associated *Leishmania* (*Sauroleishmania*) *tarentolae* Wenyon, 1921. Indeed, L. *tarentolae*, a parasite that has been detected in both sand flies and reptiles in the Mediterranean area, is regarded as nonpathogenic to mammals [4, 23–25]. Notably, experimental evidence suggests that these parasites polarize the immune response towards the Th1 side, which might ensure protection to the pathogenic *Leishmania donovani* (Laveran and Mesnil, 1903) [26]. These observations have caught the attention of researchers, who are currently investigating *L. tarentolae* for the development of novel anti-*Leishmania* vaccines [27–29]. To date, the ecology and epidemiology of this parasite remains poorly understood, and little is known on the potential pathogenicity of *L. tarentolae* to mammals and other related saurian-associated *Leishmania* parasites. For example, some *L. tarentolae* strains can cause transient infections in laboratory conditions, and one case of systemic spread of *L. tarentolae* has been described in a human mummy [30, 31]. *Leishmania* (*Sauroleishmania*) *adleri* Heisch, 1958, a lizardassociated parasite from sub-Saharan Africa related to L. *tarentolae*, has been reported to cause infections in rodents [32] and humans [33]. Moreover, sequences of an unclassified *Leishmania* species detected in CL and VL cases in China showed a close relationship to L. *tarentolae*, suggesting that a similar parasite may be involved in the disease [34, 35]. Furthermore, other studies have indicated the presence of *L. tarentolae* DNA in dogs, humans, and in sand flies of the species *P. perniciosus* in central and southern Italy [23, 36]. The detection of *L. tarentolae* genetic material in *P. perniciosus* is in accordance with the fact that *L. tarentolae* has been reported to develop in *Phlebotomus* sand flies under laboratory conditions [37]. Moreover, laboratory-maintained *S. minuta* specimens readily bite humans [38], while wild *S. minuta* have been reported to feed on humans and other mammals in Spain and Italy [39, 40]. On the other hand, *L. infantum* DNA has been detected in *S. minuta* collected in the field [36]. The two parasites and their corresponding vectors can be found in sympatry [24, 36], and coinfection by both *Leishmania* species has been observed serologically in dogs and molecularly in sand flies [24]. *Leishmania* spp. DNA has been detected in synanthropic reptiles, with *L. tarentolae* infections reported from different species of lizards and snakes in Italy. Reptiles found positive to *L. tarentolae* belonged to the species Tarentola mauritanica (Linnaeus, 1758), Podarcis filfolensis (Bedriaga, 1876), Podarcis siculus (Rafinesque-Schmaltz, 1810), Elaphe quatuorlineata (Bonnaterre, 1790), and Hierophis viridiflavus (Lacépède, 1789) [25, 41]. Moreover, in southern Italy, *L. infantum* DNA has been detected in lizards and geckos of the species Hemidactylus turcicus (Linnaeus, 1758), T.

mauritanica, and P. siculus [25], and that of L. *donovani* and *Leishmania* turanica Strelkova and colleagues 1990 in snakes and lizards from China [35, 42, 43]. Based on the current evidence, understudied herpetofauna and sand fly species may play a significant role in the emergence of *Leishmania* parasites in new regions and their maintenance in endemic areas. Given the lack of information on the occurrence of *Leishmania* spp. in the common wall lizard Podarcis muralis (Laurenti, 1768), the most common synanthropic reptile in northern Italy, and in S. minuta, we investigated the sand fly species composition and the prevalence of *Leishmania* spp. in a northern Italian district, in both sand fly vectors and synanthropic reptile hosts.

Materials and methods

Study Sites and Sand Flies Sampling.

Sand fly collections were carried out in nine peri-urban sites and three urban sites of the Bergamo district (Lombardy Region, Italy) (Figure 1). These sites comprised a variety of environmental conditions: six of the peri-urban sites were in hilly areas, characterized by the presence of deciduous vegetation, bushes, rocks, and stone walls. Livestock, housed in shelters, and domestic animals were present, as well as synanthropic reptiles. The other three periurban sites were represented by a private kennel, a private chicken coop, and a forest margin. The urban sites were in private gardens, which featured stone walls and in which synanthropic reptiles were present. A full description of the collection sites is included in Table 1.

Sand fly samplings were conducted during the sand fly activity season, between July and October 2022 and between June and October 2023. In the 2022 season, the sampling was conducted in five peri-urban sites and one urban site. Three out of five sites were again sampled in 2023, with the addition of four peri-urban and two urban sites. Sand flies were collected by setting traps one night per week. If no sand fly was collected after three sampling sessions, the site was considered negative. If a site resulted positive to the presence of sand flies, the sampling continued until there were three consecutive negative captures.

The sampling was realized employing both sticky traps (A4 papers coated with castor oil), and BG-Pro light traps in CDC configurations (Biogents, Regensburg, Germany). In this configuration, the light traps featured an LED light employed to attract insects. The light traps were also equipped with a CO2 source, the BG-Lure (Biogents, Regensburg, Germany). The sticky traps were placed inside holes and crevices of stone walls and rocks, while light traps

were settled inside animal shelters or near putative sand fly resting places (Figure 2). Both types of traps were set in the sites before sunset and retrieved the morning after. Sand flies were collected from sticky traps after being macroscopically recognized and then stored in 100% ethanol. The content of the light trap was put in a cool box. The collected insects were taken to the EntoPar laboratory of the University of Milan.

An earlier version of the study, including the sampling design and preliminary data, was presented as part of the first author's PhD thesis, submitted to the University of Milan [44].

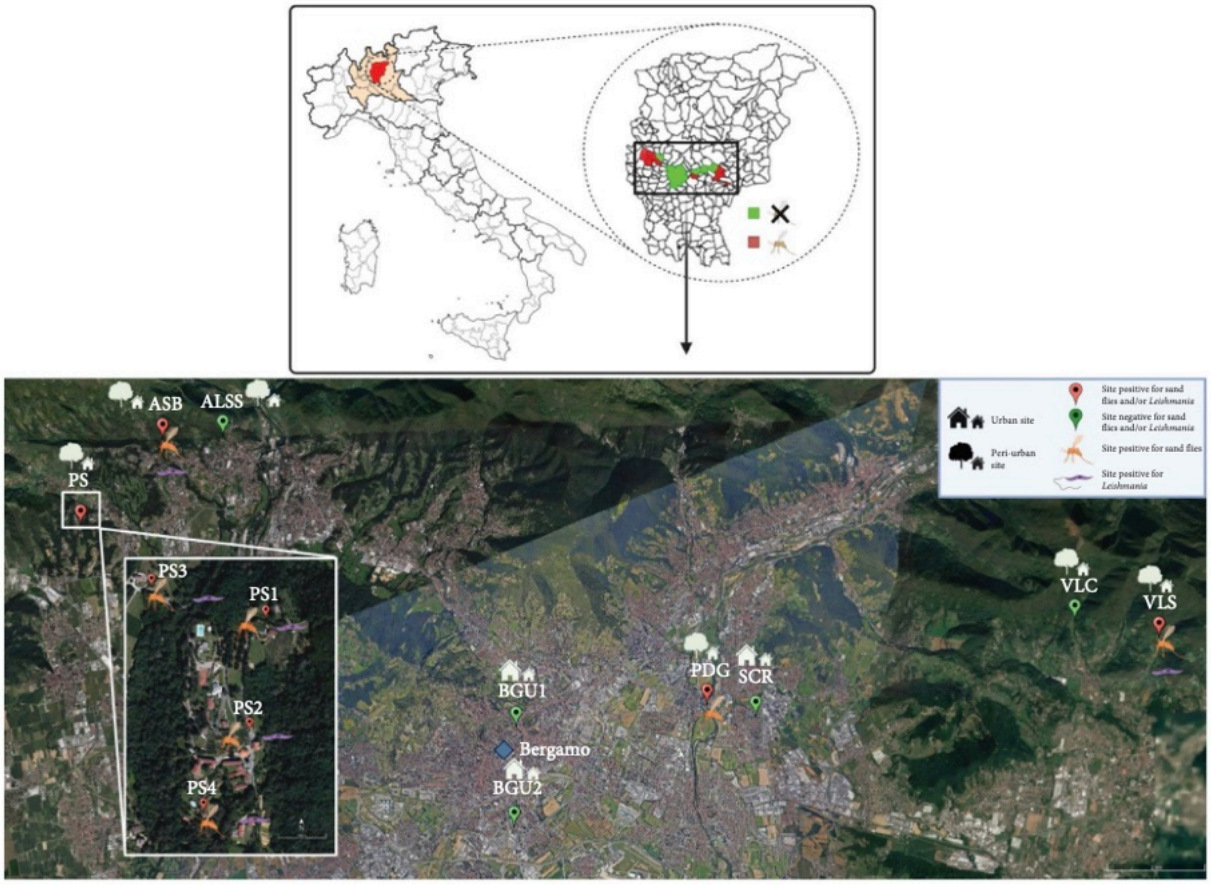

Figure 1: Location within Italy and satellite image of the sites in which sand flies monitoring was carried out.

Sand Fly Species Identification.

Once in the lab, the light traps content was put in 100% ethanol, and sand flies were isolated from other collected insects, following observation under a Leica M50 stereomicroscope (Leica Microsystems, Germany). Male sand flies were identified on the basis of the genital structure, which was dissected under the stereomicroscope and then observed under a Leica DM1000

microscope (Leica Microsystems, Germany). Species determination was then carried out following a reference morphological key [45].

Total genomic DNA was individually extracted from the whole body of each female sand fly, with no pooling of specimens. In particular, female sand flies were subjected to total DNA extraction using the DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) following the manufacturer protocol specific for insects. Female sand fly species identification was performed following a polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) protocol [46]. DNA was extracted from morphologically determined male sand flies and used as a positive control in the PCR-RFLP experiments.

Reptile Capturing and Sampling.

Synanthropic reptiles were captured by hand or using nets around the sand fly sampling sites. From reptiles, we collected the following six categories of biological samples: blood, feces, saliva, cloacal secretions, and tail tissue, and we performed blood smears. Blood was obtained via venipuncture of the ventral coccygeal vein or via cardiocentesis. A drop of blood was used to prepare a blood smear. Feces were collected after voluntary expulsion by the animal or by gently compressing their abdomen. Oral and cloacal samples were collected using cotton swabs. If dropped voluntarily during manipulation, the tail was collected and stored in 70% ethanol. After collection, all the samples were stored immediately in a cool box. After sampling, the animals were placed in a fauna box and monitored for approximately 1 h, after which they were released where they were captured. Dead animals were collected, if encountered, and placed in 70% ethanol. Once in the laboratory, all the samples were stored at −20°C.

Blood smears were stained with Giemsa and then observed under a Leica DM1000 microscope for *Leishmania* amastigotes and promastigotes detection. DNA was extracted from all the collected blood samples using the DNeasy Blood & Tissue kit following the manufacturer's protocol for animal blood and feces, respectively.

From 10 lizards, it was possible to collect at least five out of the six sample categories mentioned above. Therefore, DNA extraction was also realized from tails as well as from oral and cloacal swabs. The extraction was performed using the DNeasy Blood & Tissue kit, following the manufacturer's protocol specific to animal tissues for the tails, and the protocol for buccal swabs for the oral and cloacal swab samples.

Name	Typology	Description	Municipality	Coordinates	Years of sampling	Trap/(s)
PS1	Peri-urban	Stonewall near livestock enclosure	Palazzago	45.740338, 9.556340	2022–2023	Sticky 2022/light 2023
PS2	Peri-urban	Stonewall	Palazzago	45.738119, 9.555963	2022–2023	Sticky
PS3	Peri-urban	Stonewall	Palazzago	45.740898, 9.553106	2023	Sticky
PS4	Peri-urban	Private kennel	Palazzago	45.736697, 9.554766	2023	Light
ASB	Peri-urban	Stonewall at wood margin	Almenno San Bartolomeo	45.752813, 9.575624	2022–2023	Sticky
ALSS	Peri-urban	Wood	Almenno San Salvatore	45.754995, 9.593900	2022	Sticky
VLS	Peri-urban	Wood margin	Trescore Balneario	45.717334, 9.843545	2023	Light
VLC	Peri-urban	Stonewall near livestock enclosure	Cenate Sopra	45.720935, 9.820993	2022	Sticky
PDG	Peri-Urban	Private chicken coop	Pedrengo	45.704915, 9.723497	2023	Light
BGU1	Urban	Private garden	Bergamo	45.700605, 9.672415	2022	Sticky
BGU2	Urban	Private garden	Bergamo	45.682070, 9.671917	2023	Light
SCR	Urban	Private garden	Scanzorosciate	45.702687, 9.736528	2023	Sticky

Table 1: Information on the sites in which sand flies collection was performed

(a) (b)

Figure 2: Representative sites in which sticky traps were employed (a) and light traps were positioned (b) for the collection of sand flies.

Detection of Leishmania spp.

Female sand flies and reptile samples were screened via PCR to detect *Leishmania* spp. DNA using pan-*Leishmania* LITSR/L5.8S primers targeting a ~300 bp region of the internal transcribed spacer 1 (ITS-1) region [47]. PCR was performed in a SimpliAmp thermal cycler (Applied Biosystems, Waltham, MA). PCR reactions were carried out in a final volume of 25 µL, consisting of 5 µL of 5X GoTaq Reaction Buffer (Promega Corporation, Madison, WI), 2.5 µL of nucleotides, 0.125 µL of GoTaq G2 DNA Polymerase (Promega Corporation, Madison, WI), 1.25 of both forward and reverse primers at 10 µM concentrations, 12.875 µL of Milli-Q water, and 2 µL of DNA. *L. tarentolae* Lt-P10 strain (Jena Bioscience, Jena, Germany) and *L. infantum* MHOM/ TN/80/IPT-1 strain (WHO) DNA extracted from laboratorymaintained clones was used as a positive controls, while MilliQ water was added instead of DNA in the negative control. The PCR thermal protocol consisted of an initial denaturation step at 98°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s and annealing at 72°C for 15 s, and a final elongation step at 72°C for 10 min.

PCR products were visualized in a 1.5% agarose gel, and bands with an adequate weight were purified using the Monarch DNA Gel Extraction Kit (New England BioLabs, Ipswich, MA). The

purified products were sequences in both directions with Sanger technology (Eurofins Genomics GmbH, Konstanz, Germany).

Droplet Digital PCR (ddPCR) Screening of Engorged Sand Flies and Synanthropic Reptile's Blood for L. tarentolae and L. infantum.

The engorged sand flies and the blood collected from synanthropic reptiles were screened via ddPCR for the simultaneous detection of *L. tarentolae* and *L. infantum* kinetoplast DNA (kDNA), following the protocol described in Alvaro et al. [48]. This method employs a single primer pair to amplify a 53 bp kDNA fragment from both *L. tarentolae* and *L. infantum*, and two species-specific TaqMan probes enabling their differentiation. During the assay validation, standard curves were generated by spiking dog blood with DNA extracted from known numbers of cells of *L. tarentolae* and *L. infantum* reference strains. This allowed for an estimation of the number of parasite cells infecting the tested sand flies and reptile blood samples, by comparing their ddPCR target concentrations with the ones resulting from the above-mentioned standard curves.

Engorged Sand Flies Blood Meal Determination.

Blood meals of engorged female sand flies were molecularly determined by PCR targeting a fragment (~350 bp) of the vertebrate cytochrome b (cyt b) mitochondrial gene, using universal vertebrate primers cyt bb1/cyt bb2, as in Radrova et al. [49]. The PCR mix, the electrophoresis conditions, and the product purification procedures were the same as those adopted during *Leishmania* spp. detecting experiments of sand flies.

Phylogenetic Analysis.

The sequences generated in the present study were subjected to an nBLAST search. We downloaded the resulting hits (consisting mostly of *Leishmania* sp. sequences), along with all *L. tarentolae* hits, as well as Chinese *Leishmania* and *L. adleri* sequences. One reference sequence each for *Leishmania* tropica Wright, 1903, *Leishmania major* Yakimoff & Schokhor, 1914, *L. infantum*, and *L. donovani* was included as an outgroup. The abovementioned sequences accession numbers are reported in Table S1. All the sequences were aligned with MUSCLE, version 3.8.425 [50], within the Aliview software [51]. For the Maximum Likelihood (ML) phylogenetic analysis, all the BLAST hits, along with the selected outgroups, were

analyzed using MEGA, version 11.0.10 [52]. The K2 + G substitution model was chosen after a BIC-score analysis of nucleotide substitution models. A number of 1000 bootstrap replicates were computed. The resulting phylogenetic tree was edited using the TreeViewer software [53].

Site name	*P. perniciosus*			*P. neglectus*			*S. minuta*			TOT	Trap used
	M	F	TOT	M	F	TOT	M	F	TOT		
PS1	67	26	93	13	6	19	59	65	124	236	Sticky + light
PS2	2	2	4	8	2	10	159	106	265	279	Sticky
PS3	6	2	8	4	0	4	4	3	7	19	Sticky
PS4	10	1	11	4	0	4	0	0	0	15	Light
ASB	16	1	17	1	0	1	24	21	45	63	Sticky
VLS	21	16	37	22	4	26	0	3	3	66	Light
PDG	0	0	0	1	0	1	0	0	0	1	Light
TOT	122	48	170	53	12	65	246	198	444	679	—
ALSS	0	0	0	0	0	0	0	0	0	—	Sticky
VLC	0	0	0	0	0	0	0	0	0	—	Sticky
BGU1	0	0	0	0	0	0	0	0	0	—	Sticky
BGU2	0	0	0	0	0	0	0	0	0	—	Light
SCR	0	0	0	0	0	0	0	0	0	—	Sticky

Table 2: Total number of sand flies collected and trapping method used for every site.

Results

Sand Fly Collection.

Sand flies were found in 7/12 sites, and a total of 679 specimens were collected during the study (Table 2). Sand flies were identified as *P. perniciosus*, *P. neglectus*, and *S. minuta*. A total of 170 *P. perniciosus* (122 males and 48 females), 65 *P. neglectus* (53 males and 12 females), and 444 *S. minuta* (246 males and 198 females) were collected. The numbers of collected sand flies for each site are indicated in Table 2.

Reptile Samples Collection.

In total, 54 synanthropic specimens of *P. muralis* were captured between 2022 and 2023 in the peri-urban "PS1", "PS2", "PS3", and "PDG" sites. A total of 35 blood samples, 41 fecal samples, 10 salivary swabs, 10 cloacal swabs, and 10 tissue samples were collected. A number of 35 blood smears were performed. The collection of at least four out of the five acquirable kinds of samples from the same specimen was possible in 10 lizards.

PCR Detection of Leishmania spp. in Sand Flies and Synanthropic Reptiles.

We found *Leishmania* spp. positive sand flies at all sites where sand flies were collected, except for "PDG", where we collected only a single male. Considering all the collected females, 22.48% (58/678) resulted positive to *Leishmania* spp. by ITS-1-targeted PCR. Specifically,

31.25% (15/48) of *P. perniciosus*, 16.67% (2/12) of *P. neglectus*, and 20.71% (41/198) of *S. minuta* females resulted positive to *Leishmania* spp. (Table 3). For reptile samples, 80% (28/35) of blood samples, and 31.71% (13/41) of fecal samples (considering all the sites of reptile sampling) resulted positive to *Leishmania* spp. DNA after the ITS-1targeted PCR. In general, 62.96% (34/54) of collected individuals resulted positive to *Leishmania* spp. at least for blood or faeces. Regarding lizards from which both blood and faeces were collected, 27.78% (5/18) resulted positive to *Leishmania* spp. in both sample categories. A subset was constituted by lizards from which it was possible to collect at least four out of five kinds of samples. In this subset, all the blood samples and tissue samples resulted positive to *Leishmania* spp. DNA after PCR, while 9/10 fecal and salivary samples and 8/10 cloacal samples resulted positive to *Leishmania* spp. DNA after PCR. A number of 6/10 individuals resulted positive to *Leishmania* spp. DNA after PCR. A total of 50 ITS-1 sequences were generated from the PCR products, 35 obtained from sand flies and 15 from *P. muralis* lizards (Table S2). Moreover, one sequence was generated from the Lt-P10 strain (Jena Bioscience, Jena, Germany), maintained in the EntoPar laboratory. 3.4. ddPCR Detection of *Leishmania tarentolae* and *Leishmania infantum* in Engorged Sand Flies and Synanthropic Reptiles Blood. Following ddPCR screening of the engorged sand flies for L. *tarentolae*, 91.89% (68/74) exhibited a signal exceeding the threshold of four positive droplets, as reported by Alvaro et al. [48]. Based on the ddPCR assay protocol cited above, 4.41% of sand flies were estimated to harbor between 25 and 250 *L. tarentolae* cells, 25% contained five to 25 cells, 16.18% had one to five cells, and 54.41% carried fewer than one *L. tarentolae* cell. The frequency of *L. infantum* detection was 13.33% (2/15) in P. *perniciosus*, 66.67% (2/3) in P. neglectus, and 5.45% (3/55) in *S. minuta* engorged females. Overall, *L. infantum* was detected 9.46% (7/74) of the engorged females. In all the positive samples, the estimated parasite load was less than one *L. infantum* cell. Six out of seven sand flies positive to *L. infantum* were also positive to L. *tarentolae*, while one resulted positive to *L. infantum* only. Regarding reptiles, 97.14% of the blood samples (34/35) exceeded the positivity threshold after ddPCR screening for L. *tarentolae*. Of these samples, 2.94% were estimated to harbor between 5 and 25 *L. tarentolae* cells, 29.41% contained 1–2.5 cells, and 67.65% had 1–5 *L. tarentolae* cells. After ddPCR screening for *L. infantum*, 8.57% of blood samples (3/35) resulted positive. As for sand flies, the estimated *L. infantum* load was less than one parasite cell in every positive sample.

Species	N. F sand flies	N. F Leish+	F Leish+ (%)
2022			
Phlebotomus perniciosus	12	1	8.33
Phlebotomus neglectus	2	0	0
Sergentomyia minuta	84	10	11.90
TOT	98	11	11.22
2023			
Phlebotomus perniciosus	36	14	38.89
Phlebotomus neglectus	10	2	20
Sergentomyia minuta	114	31	27.19
TOT	160	47	29.38
2022 + 2023			
Phlebotomus perniciosus	48	15	31.25
Phlebotomus neglectus	12	2	16.67
Sergentomyia minuta	198	41	20.71
TOT	258	58	22.48

Table 3: *Leishmania* spp. prevalence values in all female sand flies obtained after ITS-1 targeted conventional PCR.

Engorged Sand Flies Blood Meal Determination.

Host DNA was amplified in 36.11% (26/72) of the engorged female sand flies. Of these, 42.6% (23/54) *S. minuta* females fed on P. muralis, nine of which resulted positive for *Leishmania* spp. after PCR; 13.33% (2/15) of *P. perniciosus* females fed on sheep, while one fed on a horse. None of the three *P. perniciosus* resulted positive for *Leishmania* spp. after PCR. Host DNA wasn't successful for any of the three engorged female P. neglectus, although one resulted positive for *Leishmania* spp. after PCR.

Reptiles Blood Smear Observations.

The observation of both amastigote and promastigote-like forms was reported in one out of 35 blood smears. The blood smear belonged to a P. muralis specimen for which *Leishmania* spp. DNA was molecularly detected in every sample category (Figure 3).

Phylogenetic Analysis.

The best BLAST hits of the sequences generated in our study are entries from organisms indicated as *Leishmania* sp., generated from *S. minuta* from Spain (accession numbers: MK567803.1, MK567795.1, LC216356.1, MK567798.1, MK567806.1, LC216368.1, MK567786.1, and MK567800), and a domestic dog from Iran (Accession Number: MT302155.1). All the above entries share high levels of similarity (97.9%–99.7%), and the ones derived from *S. minuta* have been indicated as related to *L. tarentolae* [40, 54]. Similarly, the percentage of

identity of the sequences we generate with L. *tarentolae*, as determined through BLAST search, varied from 91.49% to 98.50%. In contrast, the sequences generated in our study, as well as those mentioned above, showed a lower percentage identity with L. *adleri* (generally <90%) compared to L. *tarentolae*. We therefore anticipate that there is a group of entries in the data bases that are indicated as *Leishmania* sp. but could possibly be reclassified as *L. tarentolae* (see below and discussion), and that show high similarity with the sequences generated in this study.

Following the phylogenetic analysis, the bootstrap consensus tree (Figure S1) revealed a lack of genetic structuring among the *Sauroleishmania* sequences, which include the ones generated in this study and the BLAST hits included in the analysis, as well as the L. *adleri* sequences, resulting in most of the nodes in being unresolved. Nevertheless, the L. *adleri* sequences were clearly distinct and grouped into a monophyletic clade (with 98% bootstrap support). The outgroup, that is the sequences of the subgenus *Leishmania*, formed a monophyletic clade (bootstrap support: 99%).

In the tree with the ML (Figure 4), all the sequences generated in this study formed a monophyletic clade with GenBank entries belonging to *L. tarentolae* and with other entries in GenBank deposited as *Leishmania* sp., in agreement with the results of BLAST search (see above). As for GenBank entries deposited as L. *tarentolae*, these originated from sand flies (S. minuta, Sergentomyia dentata (Sinton, 1933), reptiles (P. siculus, T. mauritanica), and mammalian hosts (human and domestic dog) coming from southern Italy, Spain, and Turkey (Table S1). Sequences of the laboratory-maintained *L. tarentolae* strains P10 (among them, a sequence generated in the present study) and M2 cluster in this clade. These cultured isolates are kept in Italy, Iran, and the United States.

The *Leishmania* sp. sequences that clustered in this clade come from *S. minuta* screened in Spain and Portugal. The three available L. *adleri* sequences formed a monophyletic clade sister to the *L. tarentolae* plus *Leishmania* sp. clade, while the Chinese *Leishmania* sp. sequences formed a group basal to the clade composed by *L. tarentolae* plus *Leishmania* sp. and the L. *adleri* clade.

Discussion

The data from the present study provides the first evidence of the existence of an autochthonous cycle of *Sauroleishmania* in northern Italy, being the parasite DNA detected in

both sand flies and in common wall lizards hosts. Given the significant sequence similarity of the sequences generated in our study with those related to L. *tarentolae*, along with their phylogenetic placement, we can confidently conclude that the northern Italian sequences presented here belong to a species of the subgenus *Sauroleishmania*. Moreover, since the sequences generated in our study don't segregate from the sequences of *L. tarentolae* after phylogenetic analyses, they may be considered as belonging to the *L. tarentolae* species, also after taking into account their geographic origin and the host from which they derive. In addition, we also suggest that the *Leishmania* sp. entries, that we downloaded from GeneBank to perform phylogenetic analyses, should be attributed to L. *tarentolae*, for the same reasons. Indeed, another piece of evidence supporting this finding is that these sequences primarily originated from S. minuta, the confirmed vector of the parasite, sourced from countries where *L. tarentolae* is endemic.

A finer-scale and bootstrapped-supported structuring within the *Sauroleishmania* clade could not be resolved due to the high sequence similarity observed across the ITS-1 region (Figure S1) Therefore, other molecular markers should be employed to uncover potential intraclade divergence patterns that are not detectable with ITS-1 alone.

As expected, we detected *L. tarentolae* in S. minuta, the recognized sand fly vector of reptile-associated *Leishmania*. The overall *L. tarentolae* prevalence in sand flies in this study, considering all sites and species, was 20.71% based on ITS-1targeted PCR. This finding aligns with the results of Gonzalez et al. [40] in Spain, where the parasite referred to as a *Leishmania* sp. in their publication had an 18% prevalence in 377 tested *S. minuta* sand flies using the same molecular assay. In Portugal, however, the prevalence of the parasite (there identified as *Leishmania* sp.) was considerably lower, with only two out of 1867 *S. minuta* positive to *L. tarentolae* DNA by amplification of the same target [55].In southern Italy, a 12.62% prevalence of *L. tarentolae* DNA was recorded in the same sand fly species by qPCR [24].

The ddPCR results showed a higher prevalence of *L. tarentolae* (97.14%) than the one obtained after PCR in the tested engorged sand flies and revealed the occurrence of *L. infantum* DNA in the same sand flies. This could be attributed to the greater sensitivity and specificity of the ddPCR assay, which targets a conserved region of the kDNA minicircles, compared to the conventional PCR that focuses on the ITS-1 region. In fact, the ddPCR was able to detect even very low concentrations of *Leishmania* DNA, corresponding just to the parsasites "molecular signatures". A molecular signature detected from less than a single *Leishmania* cell may arise

under several scenarios, such as (i) early-stage infection, where the pathogen load is still minimal, and the infection has not yet fully established itself; (ii) environmental acquisition, where the molecular signature might be acquired due to proximity of and mere contact between infected sand flies, such as during prediuresis events, rather than reflecting an actual infection in the host, and (iii) residual presence from past infection, where the signature could result from a previous infection that occurred long ago, leaving behind only traces of molecular evidence without any active infection.

The *S. minuta* collected during our work showed a pure herpetophilic behavior, as the vertebrate DNA amplified from them belonged to P. muralis, with no evidence of feeding on mammals and humans, as reported in other studies [24, 39, 40, 55]. We also detected *L. tarentolae* in P. *perniciosus*, confirming the previous findings of studies carried out in southern Italy [24, 36] and in accordance with vector competence studies on this species [37]. The prevalence of *L. tarentolae* in the tested P. *perniciosus*, assessed with conventional PCR, was 31.25% considering all the sites, the highest recorded to date. In other studies, in southern Italy, prevalence of *L. tarentolae* in *P. perniciosus* was reported to be less than 10% [24, 56]. Moreover, we detected *L. tarentolae* DNA for the first time in P. neglectus, with an overall prevalence of 16.67%. However, *P. perniciosus* fed on horse and sheep and not on P. muralis, while it wasn't possible to amplify host DNA from engorged P. neglectus females. It may be possible that the *P. perniciosus* and P. neglectus of the sampled populations are characterized by an opportunistic feeding behavior, feeding on the blood of both mammals and reptiles. Strong evidence for this scenario is the high prevalence of *L. tarentolae* DNA detected in *P. perniciosus* and P. neglectus. Both the *P. perniciosus* and the P. neglectus specimens of the studied areas could be competent in the transmission of *L. tarentolae* to both reptiles and mammals. More sand fly samples are needed in order to elucidate the feeding preferences of sand flies in the study area. Moreover, samples of mammals living in the sand fly areas need to be screened to better comprehend the biological cycle of the *L. tarentolae* parasites detected in the study.

In the peri-urban "VLS" site, specimens of both *P. perniciosus* and P. neglectus were found positive to *L. tarentolae* DNA, with only three *Leishmania* spp. negative *S. minuta* females collected in the site. This may be due to the fact that in the "VLS" site sand flies were captured only with a light trap, which may not be attractive for S. minuta. Evidence for this hypothesis comes from the sampling results of the "PS1" site, where sticky traps were used to capture

sand flies in 2022, and in 2023 they were alternate with light traps. No *S. minuta* specimens were collected using light traps. This may explain the limited number of *S. minuta* collected with light traps in the "VLS" site.

Furthermore, our study reports the first detection of *L. tarentolae* DNA in P. muralis; the high prevalence uncovered in blood (>70%) and fecal samples (>30%) suggests that P. muralis is an important reservoir for L. *tarentolae*. The prevalence in the analyzed P. muralis population is higher than that recorded for reptiles collected in southern Italy [25] and is comparable with prevalence values recorded in China [35]. Despite this high prevalence of *L. tarentolae* in the lizard's blood, as revealed by PCR, we observed promastigote- and amastigote-like cells only in one blood smear, out of the examined 35 samples. Interestingly, we detected L. tarentoale DNA also in tissue samples and in salivary and cloacal swabs. The detection of *L. tarentolae* DNA in both blood and feces, and in other gut-associated secretions (saliva and cloacal secretions), is congruent with the idea that transmission of *Leishmania* spp. in reptiles might occur through: (i) the bite of infected sand flies; (ii) after the ingestion of sand flies; (iii) following the exposure to prediuresis secretions produced by sand flies [57]. The high prevalence of parasite DNA in lizard blood combined with the finding that *S. minuta* feeds on P. muralis indicates that inoculation of *L. tarentolae* via sand fly bite is likely a major mode of transmission. This hypothesis is also heavily supported by the results obtained after screening the samples with a more sensitive ddPCR assay, in which >90% of the blood samples resulted positive to L. *tarentolae*. The fact that only one blood smear revealed the presence of *Leishmania*-like cells in erythrocytes may be due to the ability of the parasite to colonize internal organs in the reptile host. This is in accordance with the detection of *L. tarentolae* DNA in organs of P. siculus in Southern Italy, although *L. tarentolae* was not detected in blood, feces and tails samples [24]. However, the detection of *L. tarentolae* DNA in feces and cloacal and oral swabs supports the possibility of a transmission of the parasite via ingestion by reptiles of infected sand flies. On the other hand, positivity of cloacal and oral swabs may also reflect the ability of *L. tarentolae* to visceralize in the reptile host, as in dogs positivity of conjunctival swab is associated with visceralization [58]. Finally, evidence supporting the transmission of *Sauroleishmania* parasites through the prediuresis of infected sand flies may be found in the positivity of tissue samples. These samples, which often harbor skin lesions, could serve as entry points for the parasites introduced via sand fly p rediuresis secretions. Based on the

evidence discussed above, we believe that the transmission of *L. tarentolae* through the bite of an infected sand fly is the most important and likely route.

We captured the lizards examined in this study in the same holes in which we collected sand flies with sticky traps. In addition, we also found shed lizard skins on the sticky traps. In this context, the sand flies could feed on the lizards resting inside the abovementioned holes during the evening hours, while the lizards could prey on sand flies during the morning and afternoon, when sand flies are inactive and stay inside the holes. Therefore, our results and observations are thus congruent with the existence of at least two modes of transmission of *Sauroleishmania* in reptiles; via sand fly bite, and after sand fly ingestion. This vector–host scenario, reflecting a close spatial relationship between sand flies and lizards, may also explain the high prevalence of *L. tarentolae* in lizard blood samples.

Numerous private properties present in the areas of sand fly sampling were characterized by the presence of dogs and cats. These pets may be exposed to *L. tarentolae* by being bitten by infected sand flies or by predating infected synanthropic lizards. Whether *Sauroleishmania* spp. in dogs and cats cause any pathology [30–33], or have some positive effect, such a stimulation of the immune response, that might be protective against canine and feline leishmaniasis [26], deserves further investigations [27]. On the other hand, since there is evidence that reptiles can host *Leishmania* spp. pathogenic to mammals [25, 43], predation on synanthropic reptiles may represent a risk factor for the acquisition of a *L. infantum* infection. Indeed, we detected DNA of *L. infantum* in the blood of the lizards of the sampled sites, following a ddPCR screening of the samples. The parasite DNA was found with a prevalence of 8.57% in P. muralis blood samples. This value is comparable with the 5.5% prevalence observed in P. siculus of southern Italy, although this result was obtained after testing samples of different tissues [24]. The concentrations of the parasitic DNA were very low and corresponded to less than one parasite cell. This is consistent with the following hypotheses: i) *L. infantum* may establish only transient infections in the lizards, which are not permissive reservoirs for the parasite; ii) *L. infantum* DNA might be inoculated to the lizards by *S. minuta* sand flies, which, despite having been found molecularly positive to *L. infantum* DNA in different reports, are still not regarded as a competent vectors of the parasite, and iii) blood may not be the ideal sample for *L. infantum* detection in lizards, as in the case of dogs [58]. *Leishmania infantum* is the causative agent of zoonotic VL leishmaniasis in mammals, with evidence also indicating its tropism for reptiles. The role of reptiles as reservoirs for *L. infantum*

remains unclear; however, these animals may hold significance as “sentinels” for the presence of the parasite.

By employing the ddPCR method, we also report the detection of *L. infantum* DNA in engorged sand flies from the sites “PS1”, “PS2”, and “VLS”. This is, to the best of our knowledge, the first published report of the detection of this parasite in sand flies in the Bergamo district and in the whole Lombardy region. The finding of sand flies positive to *L. infantum* in the sampling sites is in accordance with the report of two cases of autochthonous CanL in the Imagna Valley [8]. *Leishmania infantum* DNA was detected in specimens belonging to all the three examined sand fly species. In P. neglectus, two out of three engorged females tested positive to this parasite. The prevalence of *L. infantum* in *P. perniciosus* was 13,33%, (2/15 positive sand flies), while in *S. minuta* was 5.45% (3/55 positive sand flies). The *L. infantum* prevalence values for *S. minuta* are higher than those reported for sand flies in southern Italy and Portugal [59, 60].

The detection of *L. infantum* in *P. perniciosus* is congruent to the fact that the species is a proven vector of the parasite [4]. Before our current report, *L. infantum* DNA has been detected in P. neglectus from southern Italy [53]. While there is evidence for the involvement of P. neglectus in the transmission of *L. infantum* in the Balkans [7], its actual role as a vector of this parasite is still to be confirmed. The finding of *L. infantum*positive *S. minuta* is congruent with results from screening performed in central and southern Italy [24, 36]. The positivity also to *L. tarentolae* in six of the seven sand flies positive to *L. infantum* is in accordance with previous studies in southern Italy, in which positivity for the two parasites is reported in sand flies [24]. The positivity to *L. infantum* by ddPCR may be due, as discussed above, to the fact that the assay targets a conserved region of the minicircles of the kDNA, thus being more sensitive than the ITS-1-targeted conventional PCR. In fact, the concentrations of the detected parasitic DNA were very low. More samples, however, need to be collected and Sanger sequences must be generated to confirm the occurrence of this parasite in both sand flies and reptiles.

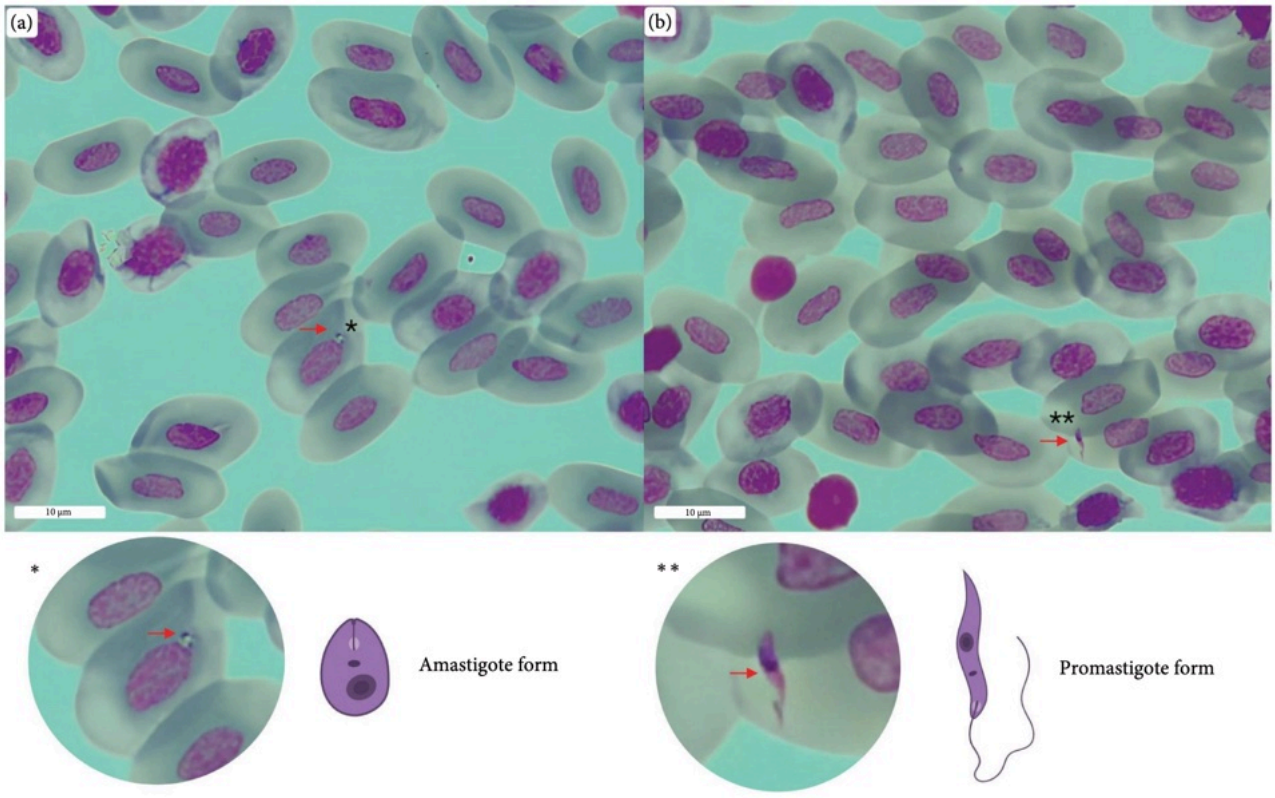

Figure 3: *Leishmania*-like parasites as observed in a blood smear of a Podarcis muralis lizard positive to *Leishmania tarentolae* after ITS-1 targeted pan-*Leishmania* PCR. (a) An amastigote-like form is seen inside erythrocytes (indicated by the red arrow). (b) A promastigote-like form is also visible (red arrow). ∗ and ∗∗ indicate parasites amastigote-like and promastigote-like forms, respectively, within the blood smears. The scale bar indicates a measurement of 10 µm.

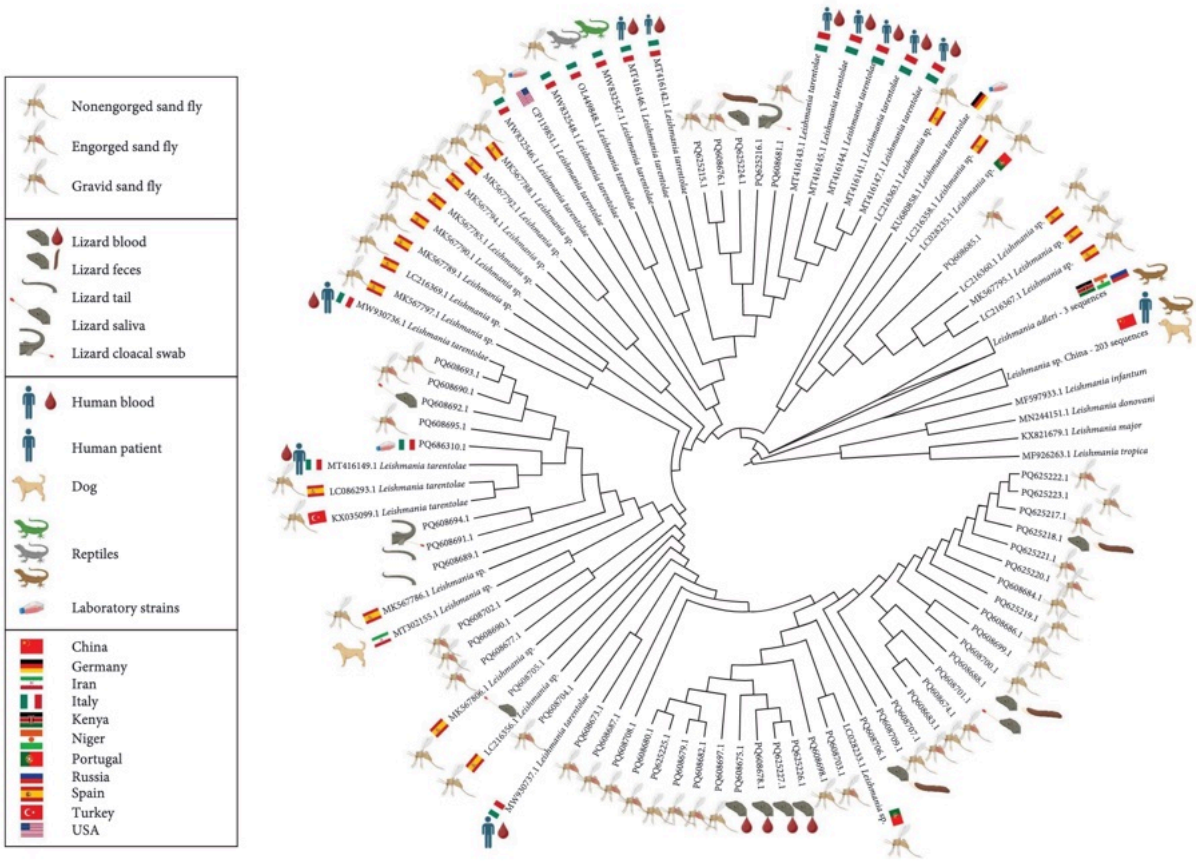

Figure 4: Tree with the highest log likelihood, according to the K2 + G nucleotide substitution model, realized with MEGA software. The substitution model was chosen after a BIC-score analysis. The origin of the sequences is indicated by icons, explained by the legend.

Conclusions

Our study contributes to the knowledge of the sand fly species composition of northern Italy, reporting the presence of *P. perniciosus*, *P. neglectus*, and *S. minuta* in the Bergamo district as well as the first report of *L. tarentolae* in northern Italy. We also report the occurrence of sand flies and reptiles positive to *L. infantum*, which is consistent with CanL cases recorded in the Bergamo district.

We emphasize that the inclusion of both *S. minuta* sand flies and synanthropic reptiles' samples in *Leishmania* spp. screenings might allow scientists to obtain a more comprehensive epidemiological picture and a better understanding of the dynamics of leishmaniasis in investigated areas.

Data Availability Statement

All the data produced during the realization of this paper are available in the paper itself and on the GenBank database.

Ethics Statement

Reptiles were captured, manipulated, and handled with approval from the Italian Ministry of Environment, as indicated by Protocol Number 77772-21/06/2022.

Conflicts of Interest

The authors declare no conflicts of interest. Funding The authors would like to acknowledge EU funding provided by the NextGeneration EU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project Number PE00000007, INF-ACT) to Claudio Bandi and Sara Epis.

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All visuals were generated using BioRender.com.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. (Supporting Information) Figure S1. Bootstrap consensus tree inferred from 1000 replicates realized with MEGA software. The nucleotide substitution model (K2 + G) was chosen after a BIC-score analysis. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap analysis are shown next to the internal branches. Each color corresponds to a specific set of sequences, as indicated by the colored squares under the tree. Table S1. Accession numbers, host, geographic origin, and strain information of L. *tarentolae*, *Leishmania* sp., *Leishmania adleri*, and outgroups ITS-1 sequences included in the phylogenetic analysis. Chinese sequences (not shown) have been obtained from the datasets of the following publications: 10.1016/j.actatropica.2016.06.023; https://doi.org/10.1371/journal.pone.0210681; 10.1007/s00436-010-1969-9. Table S2. Accession numbers, host, and source of the *L. tarentolae* ITS1 sequences generated in the study.

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7.2 *Sauroleishmania* host Diversity: New Insights from Reptilian and Amphibian Models

7.2.1 Beyond reptiles: the fire salamander as a potential host for *Leishmania* (*Sauroleishmania*) *tarentolae*

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Abstract

Leishmania parasites are dixenous protozoans transmitted by phlebotomine sand flies and known to infect a range of vertebrate hosts, including mammals, birds, and reptiles. However, to date, there is only a single record for amphibians, in a toad (order Anura), based on molecular evidence. In this study, we present the first evidence supporting the potential of *Leishmania* to infect an amphibian host, the fire salamander (order Urodela), through combined molecular and morphological approaches. A total of 78 salamanders were sampled from a protected area in northern Italy. Single cells morphologically similar to *Leishmania* were observed in 4.48 % of Giemsastained blood smears. *Leishmania*-specific qPCR coupled with high-resolution melting (HRM) analysis detected parasite DNA in 7.14 % of blood samples and 12.12 % of cloacal swabs. Sanger sequencing of a qPCR-positive sample and phylogenetic analysis identified the

parasite as *Leishmania* (*Sauroleishmania*) *tarentolae*. These findings may contribute to expand the known host range of *Leishmania* to include Urodelan amphibians, suggesting that these vertebrates may play an unrecognized role in the ecology and transmission dynamics of these parasites.

Introduction

Protozoans of the genus *Leishmania* Ross, 1903 are obligate dixenous parasites, generally transmitted by the bite of an infected female phlebotomine sand fly (Diptera: Psychodidae) to a variety of vertebrate hosts (Akhoundi et al., 2016; Cecílio et al., 2022). Mammal-infecting *Leishmania* species have received a great deal of attention due to their medical and veterinary relevance, and are included in the subgenera *Leishmania* Lainson and Shaw, 1979, Viannia Lainson and Shaw, 1987, and Mundinia Shaw, Camargo Teixeira, 2016; Espinosa et al. (2018). Species belonging to the *Leishmania* and Viannia subgenera have also been detected in birds through molecular or immunological methods (Baleela et al., 2020; Da ́valo Matheus et al., 2021; Otranto et al., 2010; Tatto et al., 2023).

Leishmania parasites belonging to the fourth subgenus *Sauroleishmania* Lainson and Shaw, 1987 primarily infect reptiles (Bandi et al., 2023; Mendoza-Roldan et al., 2022b). Parasites of the 19 valid species of this subgenus have been detected and/or isolated from reptiles of the order Squamata from the Old World (Lozano Sardaneta et al., 2018; Mendoza-Roldan et al., 2022b). In contrast to their mammal-infecting counterparts, *Sauroleishmania* parasites are generally not pathogenic, neither to reptiles nor to mammals (Bandi et al., 2023; Mendoza-Roldan et al., 2022b) with some exceptions recorded (Manson-Bahr and Heisch, 1961; Novo et al., 2015). Conversely, there is evidence that an exposure of *Leishmania* (*Sauroleishmania*) *tarentolae* Wenyon, 1921 to mammals may elicit a protective immune response towards pathogenic *Leishmania* species, by shifting it to the Th1 phenotype (Mendoza-Roldan et al., 2024; Taylor et al., 2010).

Amphibians are the only major group of terrestrial vertebrates for which unambiguous evidence of *Leishmania* infection is still lacking. So far, the only record of *Leishmania* spp. in amphibians is a recent report from the toad Rhinella horribilis (Wiegmann, 1833) from Costa Rica (Alves et al., 2025). However, this report is only based on molecular data, while analyses combining molecular and morphological data are so far lacking. This is particularly notable given that amphibians have been extensively studied in parasitology, and several groups of

hemoparasites, including trypanosomes and haemosporidians, have been well documented in their blood (Muriel et al., 2021).

In this study, we present evidence suggesting that a widespread European amphibian species, the fire salamander *Salamandra salamandra* (Linnaeus, 1758), may act as a potential host for *Leishmania*, based on an integrated approach combining morphological, molecular, and culture-based analyses. The aim of this research was to detect and identify *Leishmania* parasites in this amphibian host and to assess whether amphibians may serve as additional, previously unrecognized vertebrate hosts in the transmission cycle of these parasites.

Materials and methods

Salamander collection, handling and sampling

Fire salamanders were sampled within the regional protected area "Fontana del Guercio," a European Site of Community Interest (SCI) located in the municipality of Carugo (Como district), northern Italy. The reserve is characterized by a deciduous woodland intersected by a meandering stream and several freshwater springs, which provide suitable breeding habitats for S. salamandra (Fig. 1). Salamanders were captured during rainy nights between October and November 2022, alongside predefined transects that are part of an ongoing population monitoring program. To prevent cross-contamination, each individual was handled with a new pair of moistened, powder-free gloves. Blood was collected via puncture of the ventral caudal vein following standard protocols (Allender and Fry, 2008; Heatley and Johnson, 2009), and cloacal swabs were obtained using fine cotton-tipped applicators, gently inserted and rotated within the cloaca (Fig. 2). Blood smears were prepared using a drop of fresh blood and subsequently fixed in absolute ethanol once air-dried. The handling of and sampling from the animals were carried out under the authorization of the Lombardy region's General directorate for territory and green systems (decrees no. 18303 of December 13, 2019 and 11,721 of July 30, 2024).

Blood smears staining and microscopical observations

The ethanol-fixed blood smears were stained with Giemsa coloration. Slides were then examined under a Leica DM1000 light microscope (Leica Microsystems, Germany) for the presence of *Leishmania* amastigote and promastigote forms. Photographs were taken with the

microscope built-in camera, and parasites length was measured using the ImageJ software (Schneider et al., 2012).

Leishmania isolation attempts from salamander blood

To attempt the isolation of *Leishmania* parasites, aliquots of 30 blood samples were inoculated, right after collection, into Vacutainer tubes (Biosigma, Cona, Italy) containing Schneider's Drosophila Medium (Thermo Fisher, Waltham, USA) supplemented with 10 % foetal bovine serum (Euroclone, Milan, Italy), 1 % penicillin-streptomycin (Euroclone, Milan, Italy) and 3 mM urea. Once in the laboratory, cultures were incubated at 26 ◦C and inspected weekly by light microscopy for the presence of *Leishmania* promastigotes. Culture medium was renewed weekly for four consecutive weeks.

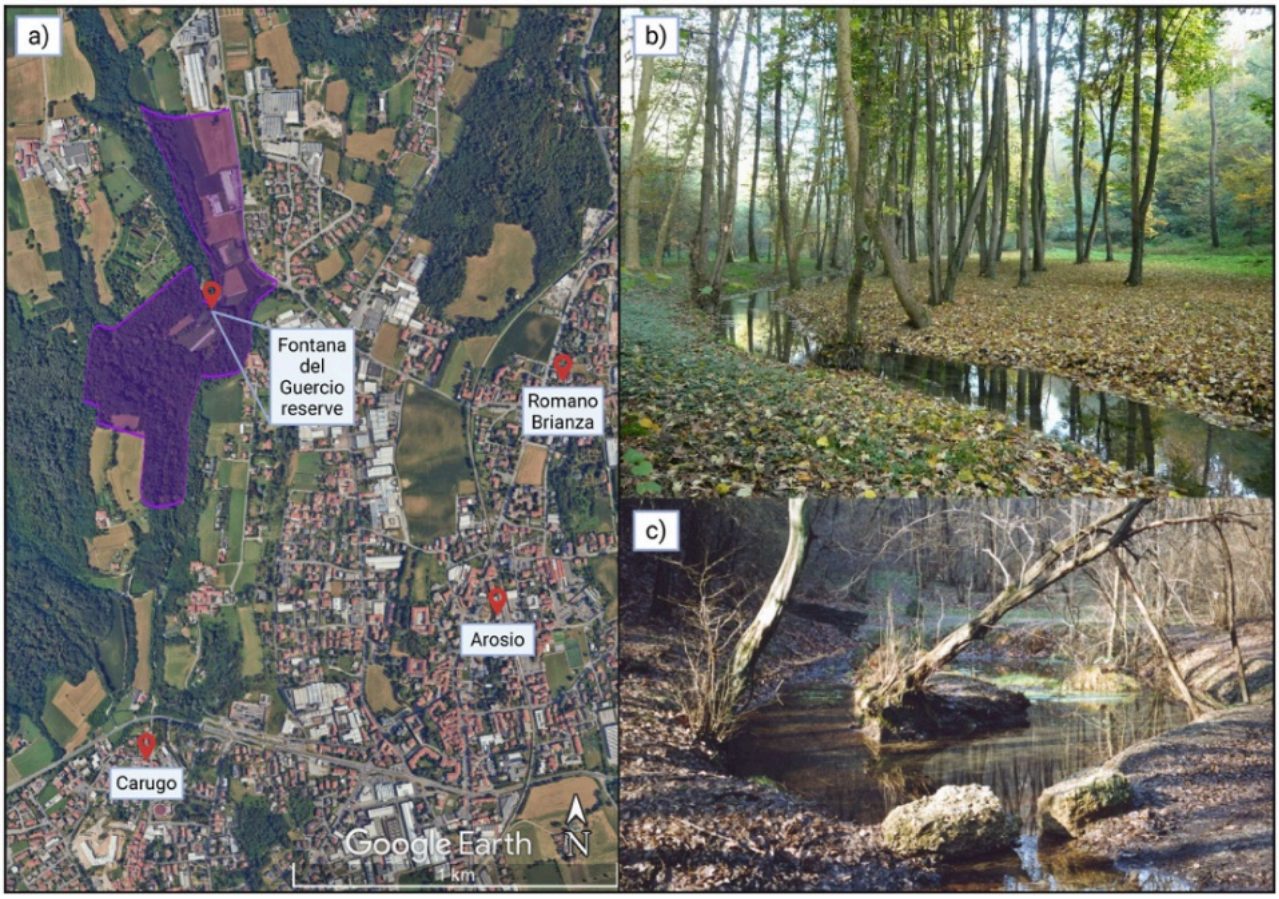

Fig. 1. Fontana del Guercio Reserve geographic position (a) and typical habitats (b,c) in which fire salamanders were collected. Reserve's shapefile downloaded from www.protectedplanet.net; photos taken from https://fondoambiente.it/luoghi/riserva-naturale-fontana-del-guercio?ldc.

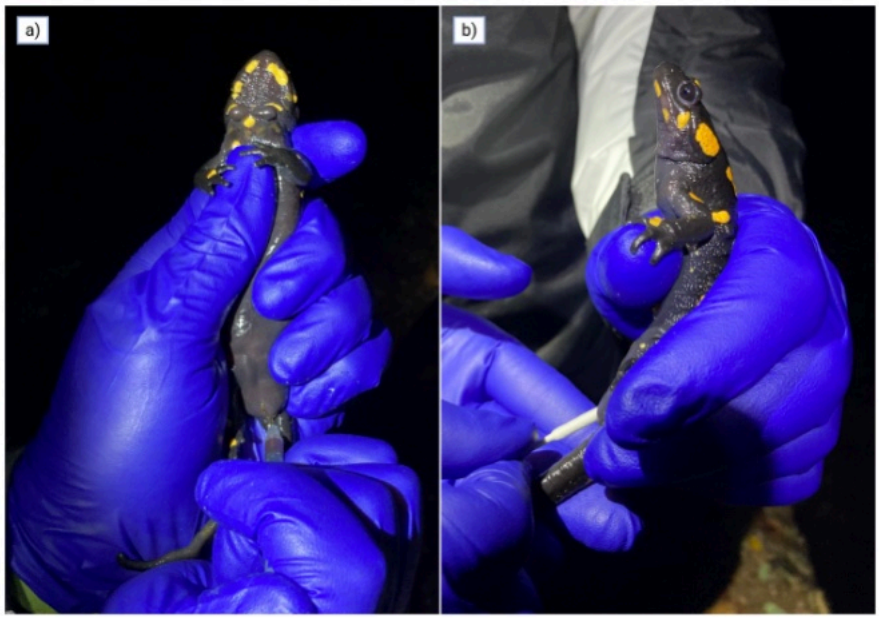

Fig. 2. Collection of blood (a) from the ventral caudal vein and a cloacal swab (b) in fire salamander individuals.

DNA extraction from blood and cloacal samples

DNA extraction was performed on the collected blood and cloacal samples using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions for animal blood and swab samples, respectively.

Leishmania spp. molecular detection

Both blood and cloacal samples were screened for *Leishmania* DNA via qPCR followed by high-resolution melting (HRM) analysis. Amplification targeted the internal transcribed spacer-1 (ITS-1) region employing pan-*Leishmania* primers LITSR and L5.8 S (El Tai et al., 2000). qPCR-HRM assays were performed on a Bio-Rad CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA). Each reaction contained 2 × SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA), primers at a final concentration of 500 nM each, 1 µL of DNA template, and Milli-Q water to bring the volume up to 20 µL. Each sample was run in duplicates. The thermal cycling protocol comprised an initial denaturation at 98 ◦C for 3 min, followed by 40 cycles of 95 ◦C for 15 s, 52 ◦C for 15 s, and 72 ◦C for 10 s. HRM analysis was then conducted by ramping the temperature from 65 to 95 ◦C with fluorescence acquisition at 0.5 ◦C increments. DNA from the commercial strain P10 of *L. tarentolae* was included as a positive control for both amplification and melting temperature analysis. Samples with melting

temperatures equal to or within ±0.5 ◦C of the P10 control, which is 83.5 ◦C, were considered positive to *Leishmania* spp. DNA. An attempt was made to amplify *Leishmania* DNA from the qPCR-positive samples by conventional end-point PCR for sequence purification, employing the same primers; however, due to unsuccess in amplification, Sanger sequencing was performed, in both directions, directly on the qPCR products (Eurofins Genomics GmbH, Konstanz, Germany).

Phylogenetic analysis

The sequence generated in the present study was subjected to an nBLAST search. The best 20 hits were downloaded together with other *Sauroleishmania* sequences. Four *Leishmania adleri* sequences were also included. One *L. infantum* sequence was added and used as the outgroup. The sequences included in the phylogenetic analysis are listed in Supplementary Table 1. All sequences were aligned into the AliView software (Larsson, 2014) using MUSCLE, version 3.8.425 (Edgar, 2004). The HKY + G4 was chosen as the nucleotide substitution model, selected using ModelTest-NG based on statistical criteria (Darriba et al., 2020). Subsequently, phylogenetic reconstruction was performed with RAxML-NG, with 1000 bootstrap replicates to evaluate the robustness of the inferred tree topology (Kozlov et al., 2019). The final phylogenetic tree was visualized and graphically refined using TreeViewer (Bianchini and Sa ńchez-Baracaldo, 2024).

Results

Sample collection

A total of 78 fire salamanders were sampled during the study. Blood samples were collected from 70 individuals, from which 67 blood smears were prepared in the field. Seven blood samples did not have corresponding smears, and, on the other hand, four blood smears lacked a matching blood sample. Cloacal swabs were taken from 66 individuals. Twelve salamanders were sampled exclusively for blood, while eight individuals only for cloacal swabs. From 58 individuals, both blood and cloacal swabs were collected and blood smears were prepared for 54 specimens.

Results of the culture-based isolation attempt of *Leishmania*

No *Leishmania* promastigotes grew in culture from any of the blood samples. Despite weekly microscopic inspections and weekly renewal of culture medium over a one-month period, no parasite proliferation was observed. After one month, all cultures were discarded.

Prevalence of *Leishmania*-like forms in blood smears

Out of 67 examined blood smears, three (4.48 %) showed the presence of *Leishmania*-like forms (Fig. 3). Of the positive smears, one (1.49 %) showed both possible promastigote and amastigote stages (Fig. 3a and b), while in two (2.98 %) only promastigotes were observed (Fig. 3c and d). In one of these two smears, the promastigote was observed undergoing binary fission (Fig. 3c). The length measurements of the identified parasites were 6.8 µm, 4.8 µm, and 6 µm for the three promastigotes, and 2.3 µm, for the amastigote.

Leishmania molecular prevalence

Considering a melting temperature of 83.5 ◦C as the reference threshold, *Leishmania* spp. DNA was detected in 5 out of 70 blood samples, resulting in a prevalence of 7.14 %. In all these cases, at least one of the replicates displayed a melting temperature of exactly 83.5 ◦C. When including samples with melting temperatures within ±0.5 ◦C of this value, the number of positive blood samples increased to 11, corresponding to a prevalence of 15.71 %.

Regarding cloacal swab samples, the number of *Leishmania* spp.positive samples, for which at least one duplicate exhibited a melting temperature of 83.5 ◦C, was eight out of 66, leading to a prevalence of 12.12 %. Expanding the threshold to include cloacal swab samples within ±0.5 ◦C of the reference value increased the number of detections to 24 samples, corresponding to a prevalence of 36.36 %.

Among the 58 salamanders from which both blood and cloacal samples were collected, only two (3.45 %) tested positive for *Leishmania* by qPCR in both sample types. In one individual, at least one replicate from each sample exhibited a melting temperature of 83.5 ◦C. In the other, at least one cloacal replicate showed a melting temperature of 83.5 ◦C, while the blood sample exhibited a melting temperature of 84 ◦C.

Only the blood sample corresponding to the smear containing both an amastigote and a promastigote tested positive for *Leishmania* spp. By qPCR; the samples corresponding to the two smears where only promastigotes were detected tested negative.

1.5. Phylogenetic placement of fire salamander-associated *Leishmania*

Only one high quality ITS-1 sequence was obtained from the qPCR products (deposited in GenBank under accession number PV762302.1). The sequence was obtained from a blood sample characterized by a melting temperature matching the *L. tarentolae* control (i.e. 83.5 ◦C). The corresponding specimen tested positive also in the cloacal swab, and the blood smear showed both the amastigote and promastigote forms. After a nBLAST search, all the first 100 hits were *Leishmania* sp. or *Leishmania tarentolae*, with percentages of identity ranging from 91.78 % to 98.68 %.

After phylogenetic analysis (Fig. 4), the novel sequence clustered in a monophyletic clade with those from *Leishmania* sp. That had been retrieved after nBLAST analysis and with *L. tarentolae* sequences (bootstrap support: 82 %). All L. *adleri* sequences formed a monophyletic clade outside of the *Leishmania* sp. plus *L. tarentolae* clade (bootstrap support: 100 %).

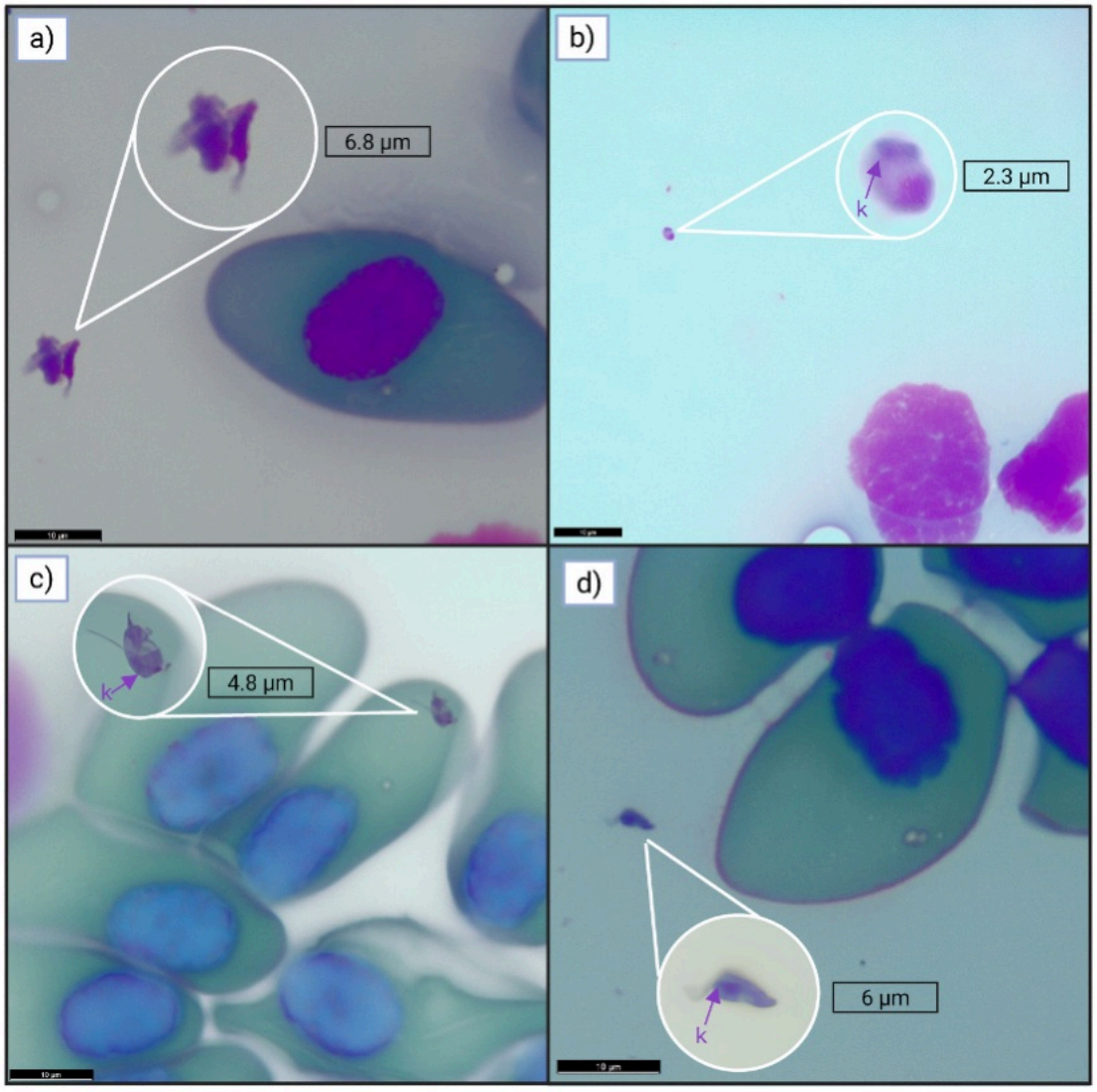

Fig. 3. Giemsa-stained blood smears showing intracellular *Leishmania*-like forms in fire salamanders blood cells. Promastigote (a) and amastigote (b) observed in the same smear. Promastigotes (c,d) from two different smears. Length measurements are indicated near the zoomed parasite images. The kinetoplast, when identifiable, is indicated by an arrow and labeled 'k'. Scale bar: 10 µm.

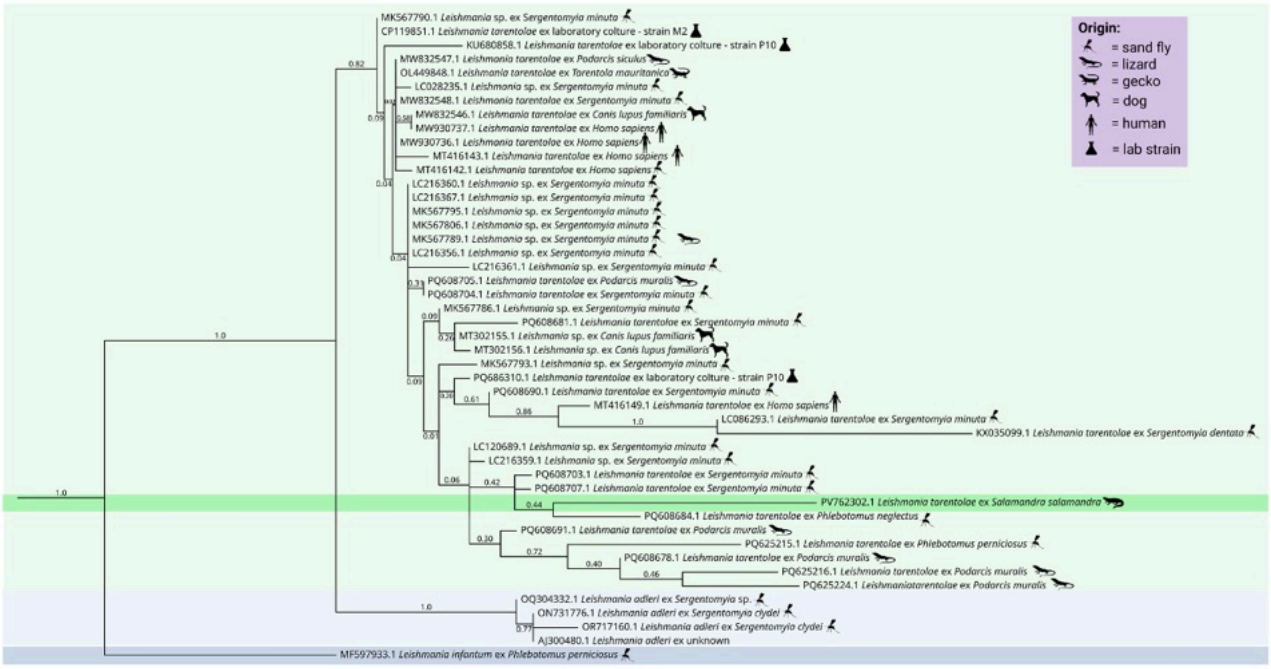

Fig. 4. Phylogenetic tree of the ITS-1 *Leishmania* sequence obtained from a fire salamander (highlighted in green). Bootstrap support values were obtained after 1000 replicates.

Discussion

In this study, we provide evidence supporting the potential role of the fire salamander (order Urodela) as a host for a *Leishmania* parasite, thereby expanding the currently known host range of this parasite genus. After phylogenetic analysis, the obtained sequence clustered in a monophyletic clade comprising sequences annotated as *Leishmania* sp. and L. *tarentolae*. The *Leishmania* sp. sequences originate from reptiles, dogs, humans, and sand flies of the species *Sergentomyia minuta* (Rondani, 1843) from Italy, Spain, Portugal, and Iran. The sequences annotated as *L. tarentolae* come from reptiles, humans and *S. minuta* sand flies from Italy, Spain, and Turkey, as well as from two laboratory strains. Moreover, the clade also includes *Leishmania* sequences obtained from our previous study conducted in the nearby Bergamo area, where we reported the first occurrence of *Sauroleishmania* in northern Italy (Alvaro et al., 2025). These sequences were generated from sand flies of the species S. minuta, *Phlebotomus*

perniciosus Newstead, 1911, and *Phlebotomus* neglectus Tonnoir, 1921, and from lizards of the species Podarcis muralis Laurenti, 1768. Since the *Leishmania* sp. sequences cluster with those of L. *tarentolae*, we believe that the sequence herein generated from a fire salamander should be attributed to the *L. tarentolae* species (Alvaro et al., 2025).

The molecular and morphological detection of *Leishmania* DNA in the blood of fire salamanders suggests that the putative infection may occur through sand fly blood-feeding on these animals. Sand flies have been observed to feed on amphibian blood (Costa et al., 2021; Netherlands et al., 2020), and amphibian DNA has been accordingly detected after molecular identification of host blood meal in engorged Sergentomyia sand flies (Shah et al., 2024). While *Sergentomyia minuta* is widely recognized as the primary vector of L. *tarentolae*, growing evidence supports the potential involvement of *Phlebotomus* species in the parasite's circulation (Alvaro et al., 2025; Mendoza-Roldan et al., 2022b; Ticha et al., 2021). In Lombardy, sand flies of the species S. minuta, P. neglectus, *Phlebotomus perfiliewi* Parrot, 1930, *Phlebotomus* mascittii Grassi, 1908, *Phlebotomus* ariasi Tonnoir, 1921, *Phlebotomus papatasi* (Scopoli, 1786) and *P. perniciosus* have been collected (Alvaro et al., 2025; Defilippo et al., 2022). The latter two *Phlebotomus* species acted as permissive vectors for *L. tarentolae* in laboratory settings (Ticha et al., 2021). Therefore, it is plausible that fire salamanders may represent a sporadic blood meal source for sand flies that are permissive vectors of *L. tarentolae* and may act as occasional vertebrate hosts in the natural life cycle of this parasite.

Although we did not carry out entomological investigations in the Fontana del Guercio reserve, sand flies are likely to occur there, as i) suitable habitat for sand flies reproduction are present (e.g. abundant leaf litter, cracks in rocks, animal burrows) (Cecílio et al., 2022), and ii) they are distributed throughout Lombardy, with *S. minuta* often representing the most abundant species (Alvaro et al., 2025; Gradoni et al., 2022).

Interestingly, although sand flies are not generally active in Lombardy in October/November, when salamander sampling took place, *L. tarentolae* DNA was molecularly detected in the blood of these amphibians. This finding aligns with evidence from blood smears, which revealed both *Leishmania*-like promastigotes, suggesting that a more recent transmission may have taken place, and an amastigote, indicating a possible intracellular replication following promastigote differentiation. This is consistent with previous reports showing *Sauroleishmania* amastigotes occupying the erythrocytes of reptiles, confirming the parasite's capacity for intracellular infection within blood cells (Alvaro et al., 2025; Mendoza-Roldan et al., 2021;

Telford, 2008). Notably, both stages were concurrently observed in one blood smear, supporting the hypothesis of ongoing parasite development within the host. The only *L. tarentolae* sequence obtained in the study came from the blood sample of the salamander corresponding to this smear; the same individual also tested positive for *Leishmania* spp. by qPCR in its cloacal sample.

Of the two smears in which promastigotes were observed, one showed a parasite undergoing binary fission, suggesting active replication. However, qPCR results for the corresponding blood samples were negative in both cases, with a melting temperature of 85.5 ◦C. This discrepancy may result from cross-reaction with non-target DNA (e.g. fungal or host) or very low parasite loads. In addition, the exceptionally large genome size of salamanders may further reduce PCR efficiency.

Salamanders possess some of the largest genomes among extant vertebrates, largely due to abundant repetitive elements and expanded introns (Weisrock et al., 2018); this may create a steric or competitive effect during amplification, particularly when parasite DNA is present in very low amounts. Still, the morphology of the promastigote-like forms observed remained consistent with that of *Leishmania*, with sizes close to the lower end of the known range for the genus (Thakur et al., 2020). Nonetheless, given the lack of detailed morphological descriptions of wild *L. tarentolae* strains (Telford, 2008) morphology provides limited taxonomic resolution.

The higher prevalence of *Leishmania* in blood samples by qPCR compared to *Leishmania*-positive blood smears, combined with the low concordance between molecular and microscopic detection, suggest that the parasite may enter the salamander blood following an infected sand fly bite and i) establish a putative self-limiting infection, eventually cleared by the host, leaving a molecular signature detectable in the blood or ii) replicate briefly in the blood before exhibiting a possible tropism for visceral tissues or intracellular localization. Both hypotheses are supported by the failed attempt to isolate *Leishmania* in culture from fire salamander blood samples.

Evidence for the first hypothesis includes reports of detection and/or isolation of *L. tarentolae* from amphibians and reptiles (Alvaro et al., 2025; Alves et al., 2025; Mendoza-Roldan et al., 2021, 2022a, 2022c).

On the other hand, evidence for the second hypothesis includes the detection of *L. tarentolae* in the viscera of lizards in southern Italy (Mendoza-Roldan et al., 2021), even though the seasonal dynamics of such infections have yet to be clarified (Mendoza-Roldan et al., 2022b).

In this scenario, the fire salamanders in which *Leishmania*-like forms haven't been observed in the blood smears may have been exposed to *L. tarentolae* during the summer, with parasites possibly persisting beyond the transmission season within visceral tissues. Conversely, fire salamanders in which the parasites were observed in their blood smears may have been exposed to the parasite at the end of the sand fly activity season (i.e. end of September/beginning of October), shortly before sampling.

On the other hand, the detection of *L. tarentolae* DNA in cloacal swabs indicates a possible transmission route of this parasite through the ingestion of infected sand flies (Alvaro et al., 2025; Mendoza-Roldan et al., 2022c). The higher percentage of positivity of cloacal samples supports the hypothesis that oral transmission may represent a more common route of exposure to *L. tarentolae* in fire salamander. In fact, the very low concordance between blood and cloacal qPCR positivity, with only two individuals out of 58 tested positives in both sample types, suggests that the two exposure routes to *L. tarentolae* might be mostly distinct. However, it should be noted that the positivity of cloacal samples may not necessarily reflect an established infection. In fact, salamanders that ingest infected sand flies may test positive for parasite DNA in cloacal swabs even in the absence of parasite replication, representing merely transient passage through the digestive tract.

Taking all the abovementioned data together, these observations suggest that fire salamanders may represent potential hosts for L. *tarentolae*, possibly being exposed to the parasite following sand fly bites, or after the ingestion of an infected sand fly. Since the biology of *Leishmania* parasites inside reptile and amphibian hosts is largely unexplored, further attempts at parasite isolation are warranted, along with systematic investigation of internal organs in both reptiles and amphibians to better understand tissue tropism, persistence, and the potential for replication in these vertebrate hosts.

Some limitations should be acknowledged in our study. The molecular detection method employed in this study targeted the ITS-1 region, a widely used marker for *Leishmania* but also a barcode commonly used for fungi, which raises the potential for cross-reactivity and falsepositive results. To minimize the risk of considering false positives, only those samples with a melting temperature strictly matching that of *L. tarentolae* (83.5 ◦C) should be considered positive. This conservative approach is likely to provide a more reliable estimate of true positivities while reducing the chance of misidentifying fungal DNA as *Leishmania*. Future work should consider the use of multiple markers and sequencing-based validation to further

refine the detection of *Leishmania* in amphibian hosts. Moreover, to obtain a more accurate estimate of *Leishmania* prevalence in the studied fire salamander population, future studies should consider the use of assays targeting kinetoplast DNA (kDNA), which is regarded as the most sensitive marker for *Leishmania* detection (Kocher et al., 2018). Another limitation of our study is that we did not sample the local sand fly vectors, which prevents us from fully characterizing the transmission cycle of *L. tarentolae* in this system. In particular, obtaining engorged sand flies testing positive to salamander DNA would have provided crucial evidence supporting the role of fire salamanders as hosts for the parasite. Future studies should also consider experimental infections of *L. tarentolae* in fire salamanders under controlled laboratory conditions to confirm whether *Sauroleishmania* parasites can establish infection in amphibians.

Urodelans have received less attention than Anurans concerning their blood parasites (Muriel et al., 2021). Instances of blood infections in salamanders caused by trypanosomes (Reichenbach-Klinke and Elkan, 1965), Haemogregarina, Pirhemocyton (Dasgupta et al., 1996), and Rickettsia bacteria (Davis and Cecala, 2010) have been documented. Although the fire salamander is widespread throughout central and southern Europe, to the best of our knowledge, very few published reports exist on its parasites other than fungi (Manenti et al., 2022; Martel et al., 2013; Spitzen-van Der Sluijs et al., 2013). Our study contributes to addressing this knowledge gap by detecting an hemoparasite for the first time in this species. Moreover, amphibians are among the most endangered vertebrates, and face multiple threats, including habitat loss and emerging diseases (Beebee and Griffiths, 2005; Stuart et al., 2004). Notably, the Fontana del Guercio fire salamander population is under further pressure due to a recently reported condition caused by an unidentified parasite (Manenti et al., 2022). Understanding and monitoring of their parasitic fauna may therefore be useful to support effective conservation efforts.

Conclusion

This study presents preliminary evidence suggesting that the fire salamander may act as a potential host for a *Leishmania* parasite, based on combined molecular and morphological methods. In doing so, we contribute to expanding the known vertebrate host range of this parasite genus and report the first hemoparasite detection in fire salamanders. Our results support the hypothesis of a possible exposure of fire salamanders to *L. tarentolae* through the

bite of an infected sand fly or via ingestion of an infected sand fly by the amphibians. Our findings highlight the possibility of including fire salamander and other amphibians as sentinels for *Leishmania* surveillance. Further research is needed to deepen our understanding of *Leishmania* infections in amphibians, Urodelan hemoparasite diversity, and to clarify the tissue tropism and the impact of a potential *L. tarentolae* infection on the endangered fire salamander population from Fontana del Guercio in particular.

CRediT authorship contribution statement

Alessandro Alvaro: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Giulia Maria Cattaneo: Writing – review & editing, Methodology, Investigation. Fabio Bigoni: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. Riccardo Molteni: Writing – review & editing, Investigation. Matilde Silvia Conconi: Writing – review & editing, Investigation. Domenico Otranto: Writing – review & editing, Supervision. Jairo Alfonso Mendoza-Roldan: Writing – review & editing, Supervision. Gentile Francesco Ficetola: Writing – review & editing, Methodology. Paolo Gabrieli: Writing – review & editing, Supervision, Conceptualization. Claudio Bandi: Writing – review & editing, Supervision, Funding acquisition. Raoul Manenti: Writing – review& editing, Methodology. Sara Epis: Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2025.101169.

Data availability

The sequence generated during the realization of the present research has been deposited in GenBank and is available under accession number PV762302.1.

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7.2.2 Unveiling *Sauroleishmania* in southern Africa: An investigation and supplementary description of *Leishmania* (*Sauroleishmania*) zuckermani from the gecko host *Chondrodactylus bibronii*

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Introduction

Parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) are the etiological agents of *Leishmania*ses, neglected tropical diseases that affect both humans and animals worldwide, ranking second only to malaria in terms of parasite-related human fatalities. In southern Africa, few cases have been reported, and data on the epidemiology and etiology of *Leishmania*ses remain scarce. Moreover, almost nothing is known about the circulation of reptile-infecting *Leishmania* parasites of the subgenus *Sauroleishmania* in the region. These parasites are generally regarded as non-pathogenic to mammals, and one species, *Leishmania* (*Sauroleishmania*) *tarentolae*, is reported to elicit a protective immune response against pathogenic *Leishmania* species in mammals upon exposure. Nevertheless, *Leishmania* (*Sauroleishmania*) *adleri* is known to have caused infections in rodents and humans. Another significant knowledge gap in southern Africa concerns sand flies, vectors of *Leishmania* parasites, recorded only from occasional, small-scale surveys conducted primarily in South Africa. In the present study, to address this gap, sand flies and Bibron's thick-toed geckos (*Chondrodactylus bibronii*) were sampled and investigated within Bankfontein in the Free State province. We provide the first report of sand flies in the Free State, and, for the first time in South

Africa, we molecularly characterized a species of *Sauroleishmania* with the supplementary description of *Leishmania* (*Sauroleishmania*) zuckermani, which has not been reported since its original description in 2001. The present study found an apparently low prevalence of the parasite, with two infected individuals of 40 geckoes screened over a three-year timeframe. One infected host was captively monitored and remained infected with the parasite for at least 415 days. Phylogenetic analysis recovered *L. (S.)* zuckermani as the sister clade of *L. (S.) adleri*. These findings highlight the need for further investigations into the diversity, distribution, and potential zoonotic risk of *Sauroleishmania* in southern Africa, alongside the need for more comprehensive sand flies' surveys.

Introduction

Leishmania Ross, 1903 (Kinetoplastida: Trypanosomatidae) is a protozoan genus of obligate dixenous parasites, primarily transmitted via the bite of infected sand flies (Diptera: Phlebotominae) to a variety of vertebrate hosts (Akhoundi et al., 2016; Cecílio et al., 2022). To date, more than 50 *Leishmania* Ross, 1903 species have been described, grouped in four subgenera: *Leishmania* Ross, 1903, Viannia Lainson and Shaw, 1987, Mundinia Shaw, Camargo and Teixeira, 2016, and *Sauroleishmania* Ranque, 1973; Akhoundi et al. (2016); Espinosa et al. (2018); Kostygov et al. (2021). The subgenera *Leishmania*, Viannia, and Mundinia include species which are the etiologic agents of the *Leishmania*ses, a group of neglected tropical diseases, which affect both humans and animals in every continent and represent the second leading cause of parasite-related mortality of humans after malaria (World Health Organisation, 2013; Savoia, 2015; Akhoundi et al., 2016). The subgenus *Sauroleishmania*, on the other hand, includes reptile-associated species, which are generally regarded as non-pathogenic to mammals (Bandi et al., 2023; Mendoza-Roldan et al., 2022b).

Sauroleishmania was first erected as a genus by Ranque (1973), with the description of cultured *Leishmania* isolated from the reptile host Tarentola annularis (Geoffroy Saint-Hilaire, 1827) from the surroundings of the Keur Tione Sarr village, in Senegal, Africa. The taxonomic rank of *Leishmania*-like haemoparasites infecting reptiles was validated by other authors but later reduced to a subgenus within the genus *Leishmania* (Kostygov et al., 2021; Klocek et al., 2023). A recent revision of the Euglenozoa taxonomy by Kostygov et al. (2021), confirmed the current status of the subgenus *Sauroleishmania* as a monophyletic sister clade to the subgenus *Leishmania*. In comparison, the subgenus *Leishmania* has received notable

attention due to its medical significance as a severe pathogen for humans and animals. Accordingly, *Leishmania* has been the target of more scientific interest and is better-studied in contrast to *Sauroleishmania*, which is still relatively understudied (Akhoundi et al., 2016; Klatt et al., 2019; Mendoza-Roldan et al., 2022b; Bandi et al., 2023; Blaizot et al., 2024).

While several species of *Sauroleishmania* infecting reptiles have been reported from sub-Saharan Africa by previous studies, as outlined by Ticha et al. (2022), only a single record is known from southern Africa, namely *Leishmania* (*Sauroleishmania*) zuckermani (Paperna, Boulard, Hering-Hagenbeck & Landau, 2001) from South Africa, reported by Paperna et al. (2001). This species was reported from the host Chondrodactylus (syn. Pachydactylus) turneri, although no molecular data accompanying the designated type material exists (Paperna et al., 2001). Furthermore, the present report is the second historical record described from southern Africa and the first confirmed report of *L. (S.)* zuckermani since the original species description. This is likely due to *Sauroleishmania* amastigote morphometrics being extremely challenging for accurate identification to species level, no available molecular data, and the lack of studies focussing on *Sauroleishmania* in South Africa.

While other species of the genus *Leishmania* are known to be zoonotic and cause devastating diseases, the zoonotic potential of *Sauroleishmania*, specifically *Leishmania* (S.) zuckermani, and its effects on the reptile host are not completely understood. On the basis of present understanding, parasites of the subgenus *Sauroleishmania* appear to be non-pathogenic to both reptiles and mammals (Bandi et al., 2023; Mendoza-Roldan et al., 2022b). Remarkably, on the other hand, studies suggest that when mammals are exposed to *Leishmania* (*Sauroleishmania*) *tarentolae* (Wenyon, 1921), the parasite may trigger a protective immune response against pathogenic *Leishmania* species by promoting a Th1-type response (Mendoza-Roldan et al., 2024; Taylor et al., 2010).

Nevertheless, some *Sauroleishmania* parasites have been reported to behave as pathogens under certain conditions. One case of a systemic spread of *L. (S.) tarentolae* has been reported from a human mummy from Brazil (Novo et al., 2015), and some strains of *L. (S.) tarentolae* caused transient infections under laboratory conditions (Taylor et al., 2010). Moreover, *Leishmania* (*Sauroleishmania*) *adleri* (Heisch,1958), a sub-Saharan African species, was reported to infect humans and rodent hosts (Adler, 1962; Manson-Bahr and Heisch, 1961). Additionally, an undescribed *Leishmania* species, related to *L. (S.) tarentolae*, was detected in leishmaniasis cases from China (Yang et al., 2010; Zhang et al., 2016).

While *Leishmania* are known to be mostly transmitted by phlebotomine sand flies (Cecílio et al., 2022) and in some cases biting midges (Diptera: Ceratopogonidae) (Becvar et al., 2021), there is still a lack of understanding about the full life cycle and uncertainty about the mechanism of transmission of some of these parasites, especially of the reptile-infecting species of the *Sauroleishmania* subgenus (Ticha et al., 2022).

Currently, *Leishmania* (*Sauroleishmania*) *adleri*, *L. (S.) ceramodactyli* (Adler & Theodor, 1929), *L. (S.) hoogstraali* (McMillan, 1965), *L. (S.) platycephala* (Telford, 2009), and *L. (S.) senegalensis* (Ranque, 1973) are other species of the *Sauroleishmania* that have been reported from sub-Saharan Africa (Killick-Kendrick et al., 1986; Telford, 2009; Akhoundi et al., 2016; Ticha et al., 2022). Of these species, molecular data is currently only publicly available on NCBI databases for the two species: *L. (S.) adleri* and *L. (S.)* hoogstraali.

Given the limited knowledge on *Sauroleishmania*, particularly in subSaharan Africa, the present conducted study was conducted with the purpose of addressing this gap in the knowledge and contributing to the scientific information from this area.

The aim of this study was to: 1) investigate if previously reported *Sauroleishmania* was still occurring in South Africa and assess the taxonomic status with morphological and molecular data; 2) explore the potential prevalence, parasitaemia, life cycle of this *Sauroleishmania* in gecko hosts; 3) screen and identify potential vectors and routes of transmission; 4) assess the infection cycle in the blood of a host over time, and attempt to isolate a haemoculture.

Materials and methods

Collection of reptile host specimens

Three separate sampling events were conducted, beginning in 2023 and ending in 2025, whereby multiple gecko host specimens (*Chondrodactylus bibronii*) were collected from several localities within Bankfontein, a field site in the Free State Province, South Africa [Table 1 and Fig. 1]. The first sampling event took place in October 2023, where five *C. bibronii* were collected. The second sampling event took place in March 2024, with ten *C. bibronii* being collected. The third and final sampling event occurred during March 2025, with 25 *C. bibronii* being collected. In total, 40 *C. bibronii* specimens were collected and examined during the present study. Adult animals were mainly targeted, with some sub-adults also being collected. Gecko specimens were captured by hand and/or using small nooses opportunistically from crevices in rocks. Specimens were examined for the presence of external parasites.

Blood was collected from the specimens via cardiac centesis or femoral venipuncture. Following the procedure described by Netherlands et al. (2015), two blood smear slides were made from each specimen, air-dried, and fixed with absolute methanol. Fixed slides were stained using a 10 % modified Giemsa (Sigma-Aldrich, Steinheim, Germany) solution. For molecular analysis purposes, excess blood was fixed in a 2 ml screw-cap cryovial containing absolute ethanol. A light microscope was available to preliminarily screen samples in the field. A single, infected gecko (RE231006C1) from the first sampling event in October 2023 was kept captive for further analysis and microscopically screened in regular intervals during the period between October 6, 2023 and March 27, 2025 (over a total of 539 days).

Morphological characterisation

Giemsa-stained blood smear slides were analysed and photographed using a Leica DFC 7000 T camera and Leica DM6 B microscope, powered by a Leica CTR6 LED power supply. Images were captured at 1000x magnification and analysed using FIJI processing package software, version 1.0.0 (Schindelin et al., 2012). Morphometrics measurements were adapted from the methodology of Paperna et al. (2001) and Telford (2009). These morphometrics included BL = amastigote body length, BW = amastigote body width, and aggregate size. The number of amastigotes within the affected erythrocyte was also noted. Morphometric measurements were taken from a total of 27 amastigotes, and seven aggregates [Table 2].

Collection of potential Sauroleishmania vectors

To assess the presence of potential vectors in the environment, sand flies were collected over a one-week period in March 2025 across various sites surrounding the Bankfontein research station, Free State province, South Africa [Table 3]. Sampling was conducted daily using two complementary methods: sticky traps (A4-sized sheets coated with castor oil) and BG-Pro light traps in a CDC-miniature configuration (Biogents, Regensburg, Germany). These traps were deployed at locations deemed suitable for sand fly resting and breeding. Site selection was based on ecological features, including proximity to hyrax dens, rock piles, and termite mounds, as well as areas with high gecko activity. They were also placed in sites close to where the *Sauroleishmania*infected geckos were captured. In one instance, traps were placed at a specific rock crevice where sand fly-like insects were observed. This collection effort aimed

solely to confirm the presence of potential vector populations to support the feasibility of a vector-borne transmission cycle for the reptilian *Sauroleishmania* infection.

Table 1 Sampling localities and information of *C. bibronii* host specimens.

Date	Field Number	Host species	Sex	Latitude	Longtitude	Elevation	Leishmania infection
2023 October 5	RE231005D1	*Chondrodactylus bibronii*	Sub-adult	S 30.073843°	E 24.883678°	1186 m	–
2023 October 6	**RE231006C1**	***Chondrodactylus bibronii***	**Adult female**	**S 30.073843°**	**E 24.883678°**	**1186 m**	+
2023 October 6	RE231006E2	*Chondrodactylus bibronii*		S 30.073843°	E 24.883678°	1186 m	–
2023 October 9	RE231009G1	*Chondrodactylus bibronii*	Adult female	S 30.073843°	E 24.883678°	1186 m	–
2023 October 10	RE231010D1	*Chondrodactylus bibronii*	Sub-adult	S 30.073843°	E 24.883678°	1186 m	–
2024 March 8	RE240308D1	*Chondrodactylus bibronii*	Adult female	S 30.073844°	E 24.883687°	1186 m	–
2024 March 9	RE240309C1	*Chondrodactylus bibronii*	Sub-adult female	S 30.073844°	E 24.883687°	1186 m	–
2024 March 9	**RE240309D1**	***Chondrodactylus bibronii***	**Adult female**	**S 30.076113°**	**E 24.880241°**	**1179 m**	+
2024 March 9	RE240309D2	*Chondrodactylus bibronii*	Adult female	S 30.076113°	E 24.880241°	1179 m	–
2024 March 9	RE240309E1	*Chondrodactylus bibronii*	Adult male	S 30.075806°	E 24.880305°	1179 m	–
2024 March 9	RE240309E2	*Chondrodactylus bibronii*	Adult female	S 30.075806°	E 24.880305°	1179 m	–
2024 March 10	RE240310F1	*Chondrodactylus bibronii*	Sub-adult	S 30.065266°	E 24.894460°	1204 m	–
2024 March 10	RE240310G1	*Chondrodactylus bibronii*	Adult female	S 30.073844°	E 24.883687°	1186 m	–
2024 March 11	RE240311D1	*Chondrodactylus bibronii*	Adult female	S 30.076418°	E 24.884172°	1181 m	–
2024 March 11	RE240311G1	*Chondrodactylus bibronii*	Adult male	S 30.076429°	E 24.881922°	1184 m	–
2025 March 8	RE250308A1	*Chondrodactylus bibronii*	Adult female	S 30.072453°	E 24.884789°	1188 m	–
2025 March 8	RE250308A2	*Chondrodactylus bibronii*	Adult male	S 30.072453°	E 24.884789°	1188 m	–
2025 March 8	RE250308D1	*Chondrodactylus bibronii*	Sub-adult	S 30.074222°	E 24.884051°	1126 m	–
2025 March 8	RE250308D2	*Chondrodactylus bibronii*	Adult male	S 30.074222°	E 24.884051°	1126 m	–
2025 March 8	RE250308E1	*Chondrodactylus bibronii*		S 30.073724°	E 24.884382°	1227 m	–
2025 March 9	RE250309A1	*Chondrodactylus bibronii*	Adult female	S 30.071260°	E 24.883511°	1186 m	–
2025 March 9	RE250309B1	*Chondrodactylus bibronii*	Adult male	S 30.074235°	E 24.883857°	1186 m	–
2025 March 10	RE250310A1	*Chondrodactylus bibronii*	Adult male	S 30.075224°	E 24.885553°	1199 m	–
2025 March 10	RE250310C1	*Chondrodactylus bibronii*		S 30.076197°	E 24.884175°	1194 m	–
2025 March 10	RE250310C2	*Chondrodactylus bibronii*		S 30.076546°	E 24.883989°	1194 m	–
2025 March 11	RE250311D1	*Chondrodactylus bibronii*	Adult male	S 30.065016°	E 24.894613°	1201 m	–
2025 March 11	RE250311E1	*Chondrodactylus bibronii*	Sub-adult	S 30.072539°	E 24.884910°	1190 m	–
2025 March 11	RE250311F1	*Chondrodactylus bibronii*	Sub-adult	S 30.073672°	E 24.886936°	1200 m	–
2025 March 11	RE250311F2	*Chondrodactylus bibronii*	Sub-adult	S 30.073672°	E 24.886936°	1200 m	–
2025 March 11	RE250311F3	*Chondrodactylus bibronii*	Sub adult	S 30.073430°	E 24.8873555°	1202 m	–
2025 March 11	RE250311G1	*Chondrodactylus bibronii*	Adult male	S 30.074516°	E 24.887044°	1118 m	–
2025 March 11	RE250311H1	*Chondrodactylus bibronii*	Adult female	S 30.075636°	E 24.885803°	1196 m	–
2025 March 11	RE250311H2	*Chondrodactylus bibronii*	Sub-adult	S 30.075636°	E 24.885803°	1196 m	–
2025 March 11	RE250311H3	*Chondrodactylus bibronii*	Sub-adult	S 30.075636°	E 24.885803°	1196 m	–
2025 March 11	RE250311I1	*Chondrodactylus bibronii*	Adult	S 30.075717°	E 24.886055°	1195 m	–
2025 March 11	RE250311I2	*Chondrodactylus bibronii*	Adult	S 30.075717°	E 24.886055°	1195 m	–
2025 March 11	RE250311I3	*Chondrodactylus bibronii*	Sub adult	S 30.075717°	E 24.886055°	1195 m	–
2025 March 12	RE250312A1	*Chondrodactylus bibronii*	Adult	S 30.074235°	E 24.883857°	1186 m	–
2025 March 12	RE250312B1	*Chondrodactylus bibronii*	Sub-adult	S 30.074707°	E 24.883264°	1187 m	–
2025 March 12	RE250312B2	*Chondrodactylus bibronii*	Sub-adult	S 30.075335°	E 24.882838°	1185 m	–

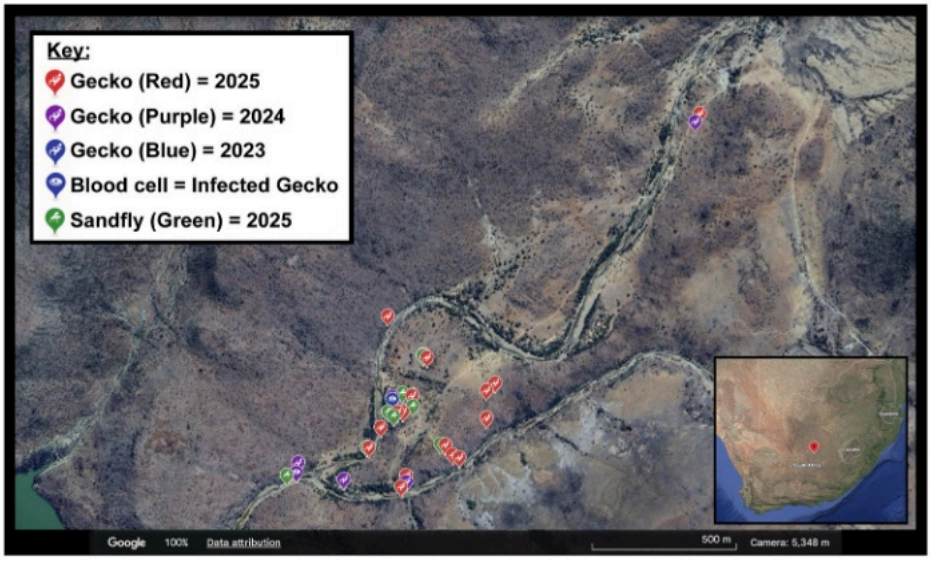

Fig. 1. Map of gecko and sand fly sampling sites within the Bankfontein study area, South Africa. Created using imagery from Google Earth. Year indicated by colour, sample type indicated by symbol.

Table 2 Morphometrics of *Leishmania* (*Sauroleishmania*) zuckermani amastigotes.

	L. (*S.*) *zuckermani* (Present study)	*L.* (*S.*) *zuckermani* (Paperna et al., 2001)	*L.* (*S.*) *platycephala* (Telford, 2009)
Amastigote BL	2.15–4.42 (3.09 ± 0.49) n = 27	2–2.2 n = 6	2–5
Amastigote BW	0.87–2.2 (1.21 ± 0.32) n = 27	1–1.5 n = 6	1–2
Aggregate size	3.11–4.86 (4.11 ± 0.67) n = 7	3–5	

Table 3 Coordinates, characteristics and sampling results for vector collection sites.

Sample site	Sand flies presence	Latitude	Longitude	Characteristics of the sample sites
BF1	Positive	S 30.074352°	E 24.883722°	Hyrax den
BF2	Negative	S 30.074230°	E 24.883527°	Rock where geckoes were observed
BF3	Positive	S 30.075197°	E 24.885375°	Rock where sand flies were observed
BF4	Negative	S 30.076200°	E 24.879894°	Near the site of a positive gecko capture in 2023
BF5	Negative	S 30.075322°	E 24.882761°	Near the site of a positive gecko capture in 2023
BF6	Positive	S 30.072483°	E 24.884769°	Termite mound
BF7	Negative	S 30.073647°	E 24.884038°	Rock pile
BF8	Negative	S 30.074041°	E 24.884400°	Rock where geckoes were observed

Morphological sorting of phlebotomine sand flies

All insects captured on sticky traps were carefully removed with a fine brush and stored in 100 % ethanol-filled vials. The light traps content was retrieved and brought to the research station. All the collected insects stored in 100 % ethanol-filled petri dishes and observed under a stereomicroscope. Phlebotomine sand flies were recognised morphologically based on the following key characteristics: small body size, long legs, hairy appearance, hairy wings with typical morphology and venation, and the presence of prominent, widely separated black eyes. Males were distinguished from females by the presence of prominent external genitalia, which are absent in females.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from all 25 *C. bibronii* blood samples from 2025 and from two infected (microscopically confirmed) hosts from 2023 to 2024, using the Quick-DNATM Miniprep Plus (Zymo Research, California, USA) extraction kit, following the manufacturer's protocol specific for nucleated blood. The extracted DNA was then subjected to PCR amplification targeting two different genetic regions: a portion of the 18S small subunit ribosomal RNA (18S rRNA) gene and a fragment of the internal transcribed spacer (ITS-1) region. The 18S-targeted PCR was performed as a nested assay, using two sets of conserved primers and following thermal cycling conditions as detailed in Jordaan et al. (2023). The PCR targeting the ITS-1 region was carried out employing pan-*Leishmania* primers (El Tai et al., 2000) and following a modified thermal cycling protocol as in (Alvaro et al., 2025). All the PCRs were performed in a SimpliAmpTM thermal cycler (Applied Biosystems, Waltham, MA). The PCR products were analysed by electrophoresis on a 1.5 % agarose gel, to assess their size. The non-purified products were sent for purification and sequencing in both directions with Sanger technology (Inqaba Biotec, Pretoria, South Africa).

Genomic DNA was extracted from all 32 female sand fly specimens using the same commercial kit employed for the gecko samples but following the manufacturer's insect-specific protocol. Subsequently, all extracts were screened for *Leishmania* DNA via PCR amplification of the ITS-1 region, utilizing the same primers and reaction conditions as those applied to the reptile hosts

Attempted culturing of parasites

Leishmania isolation was attempted from *C. bibronii* blood using Novy–MacNeal–Nicolle (NNN) medium, following the protocol described by Castelli et al. (2023). Each vial contained 4 ml of solid-phase medium (beef extract, 3 mg/ml; peptone, 5 mg/ml; sodium chloride, 8 mg/ml; agar, 15 mg/ml; pH 7.3 ± 0.2. Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % defibrinated bovine blood, overlaid with 2 ml of liquid-phase medium (potassium chloride, 0.2 mg/ml; sodium chloride, 8 mg/ml; calcium chloride, 0.2 mg/ml; anhydrous potassium dihydrogen phosphate, 0.3 mg/ml; dextrose, 2.5 mg/ml; pH 7.0 ± 0.2. Sigma-Aldrich, St. Louis, MO, USA). Blood samples collected from potentially infected reptiles during the 2025 sampling event were directly inoculated in the field into sterile vacutainers containing NNN medium supplemented with 1 % Penicillin–Streptomycin solution (Euroclone, Milan, Italy). The vials

were incubated at 26 ◦C under aerated conditions and monitored by optical microscopy observation for up to three weeks.

In addition, *Leishmania* isolation was attempted from the blood of a captive gecko specimen (identification code: RE231006C1) that had previously tested positive by molecular analysis and microscopy.

Phylogenetic analysis

The sequences generated in this study were compared with reference nucleotide sequences using nBLAST. For each sequence, the best hits were downloaded together with representative sequences of African *Leishmania* species. In addition, sequences for the two genetic markers were retrieved from all *Sauroleishmania* species available in the NCBI database. All sequences were subsequently aligned with MUSCLE version 3.8.425 (Edgar, 2004) within the AliView software (Larsson, 2014). The primer sequences were removed.

For each marker, an alignment was generated. The alignments were then subjected to phylogenetic analyses. The best nucleotide substitution model for every alignment was selected with the ModelTest-NG software, version 0.1.7 (Darriba et al., 2020), following model evaluation based on BIC scores. The HKY + I substitution model was chosen for the 18S alignment, while the HKY + G4 model was selected for the ITS-1 alignment. A Maximum-Likelihood (ML) phylogenetic reconstruction was then carried out with the RaxML-NG software, version 1.2.2 (Kozlov et al., 2019). One thousand bootstrap replicates were conducted. The final phylogenetic tree was then refined using the TreeViewer software (Bianchini and Sa ńchez-Baracaldo, 2024).

Results

Two geckoes of the total 40 sampled were found to be infected by *Sauroleishmania* sp., after microscopic and molecular analyses [see Table 1]. The overall prevalence 2/40 (4.44 %), while being 1/5 (20 %) for October 2023, 1/10 (10 %) for March 2024, and a prevalence of 0/25 (0 %) for March 2025.

An infected gecko specimen (RE231006C1) was kept captive and regularly screened approximately every 10 days (for the first 7 months after capture), less frequently thereafter. While the initial date of natural infection with the parasite is unknown to the researchers, a *Sauroleishmania* infection was observed in the screened blood smears of the host consistently

until 415 days after capture (24/11/2024), where the infection parasitaemia suddenly decreased and was only observed extracellularly, peripheral to damaged blood cells. No *Sauroleishmania* infection was observed with microscopic screening 9 days later (December 3, 2024), and subsequent screenings thereafter was not able to determine a positive infection. At 378 days (October 18, 2024), the infection was still observed to be intraerythrocytic. The infection appeared to remain relatively constant over the 415 days of infection, only being observed by the researchers inside erythrocytes or extracellularly apparently due to ruptured cells. At the final screening after 539 days (March 27, 2025), there was still no positive infection was observed in the specimen.

Diagnosis

Phylum: Euglenozoa Cavalier-Smith, 1981.

Class: Kinetoplastea Honigberg, 1963, emend. Vickerman, 1976.

Subclass: Metakinetoplastina Vickerman, 2004.

Order: Trypanosomatida Kent, 1880.

Family: Trypanosomatidae Doflein, 1901.

Subfamily: Leishmaniinae Maslov and Lukesˇ, 2012.

Infrafamily: *Leishmania*tae Maslov and Lukesˇ, 2012.

Genus: *Leishmania* Ross, 1903.

Subgenus: *Sauroleishmania* Ranque, 1973.

Supplementary description of Leishmania (Sauroleishmania) zuckermani (Paperna, Boulard, Hering-Hagenbeck and Landau, 2001) Jordaan & Netherlands, 2025

Host: *Chondrodactylus bibronii* (Smith, 1846) (Squamata: Gekkonidae: Gekkota)

Localities in this study: [RE231006C1]: Bankfontein research station, S 30.073843◦ E 24.883678◦ (1186 m), Free State Province, South Africa. [RE240309D1]: Bankfontein research station, S 30.076113◦ E 24.880241◦ (1179 m), Free State Province, South Africa. *Type host: Chondrodactylus turneri* (Gray, 1864) (Synonymous Pachydactylus turneri) (Squamata: Gekkonidae: Gekkota)

Type locality: Northern Pretoria, S 25.614166◦ E 28.001666◦ (25◦36'51'' S, 28◦00'06'' E), Gauteng Province, South Africa.

Type material: Hapantotype, 1x blood smear N◦. 225 PZ and paratype, 1x blood smear N◦. 222 PZ deposited in the collection of the Muse úm Nationald'Histoire Naturelle, Paris, France.

Supplementary material: 2 × blood smears from the type host *C. bibronii*, deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession numbers NMB P 1193 [RE231006C1] & NMB P 1192 [RE240309D1].

Representative DNA sequences: The sequence data specifically associated with *L. (S.)* zuckermani (upon which the present biological description is based) has been submitted to GenBank and are as follows: Nuclear 18S rDNA (nu 18S) partial sequences PX572415 [RE231006C1] and PX572416 [RE240309D1], Internal Transcribed Spacer (ITS1) region nuclear DNA sequences PX572417 [RE231006C1] and PX572418 [RE240309D1].

Site of infection: Peripheral blood. Stages in host: Amastigote.

Vector: Unknown. Suspected sand flies (Diptera: Psychodidae: Phlebotominae), proven vectors of *Sauroleishmania* parasites, occur in the localities of this study.

Stages in vector: Unknown. In sand flies, *Sauroleishmania* promastigotes occur.

Morphology: Measurements in micrometres (µm). Measurement range (average ± standard deviation) [Table 2].

Number of amastigotes per infected cell (erythrocyte) 1–8.

Body Length of amastigotes: 2.15–4.42 (3.09 ± 0.49) (n = 27)

Body Width of amastigotes: 0.87–2.2 (1.21 ± 0.32) (n = 27)

Size of aggregates: 3.11–4.86 (4.11 ± 0.67) (n = 7)

A single kinetoplast and nucleus were clearly visible within the parasite body [Fig. 2].

Aggregates (apparently in process of division) were observed to be comprised of between 2 and 5 amastigotes. Paperna et al. (2001) describe these aggregates containing up to 25 or more parasites. Consistent to the description of Paperna et al. (2001), round aggregates consisting of peripheral basophilic parasite bodies with a 'central hollow' were also commonly observed in the present study.

Remarks

The morphology and morphometrics in this study are identical to the description provided by Paperna et al. (2001) of *Leishmania* (*Sauroleishmania*) zuckermani [Table 2]. However, it is still challenging to distinguish species of *Sauroleishmania* based solely on morphometric characteristics. In conformity with the study of Paperna et al. (2001), only amastigote

Sauroleishmania stages were observed in the peripheral blood during this study, presenting as single cells, cell assemblies, and aggregates of cell clusters. Unfortunately, Paperna et al. (2001) did not provide molecular data from the type series material in their species description. While the description of Paperna et al. (2001) included valuable details on the ultrastructure, due to the knowledge at the time, they believed it could not be morphologically distinguished from other *Leishmania* species, even with ultrastructural comparisons. Although, current literature has shown this not to be necessarily true, as explained by Telford (2009) and supported by the findings of Lewis (1975) when comparing the ultrastructural characteristics of *Sauroleishmania* spp. to other species of *Leishmania*.

In the present study, the captive specimen (RE231006C1) monitored over the three-year period was found to be positively infected with *Sauroleishmania* amastigotes during regular screening intervals until it was no longer observed to be infected after 415 days and onwards. Between screenings on 415th day and 424th day, the infection had dissipated and was no longer detected on day 424.

During the 415 days, the infection was observed to remain relatively consistent, with an apparent slight decrease over time in the parasitaemia from the initial date of screening. On the 415th day infection parasitaemia was noticeably lower from the previous screening (37 days prior), and only a few parasites observed to be present in the slide. Furthermore, none of these amastigotes were observed intracellularly, and were on the periphery of apparently damaged erythrocytes ('smudge cells').

An apparently unique characteristic to *L. (S.)* zuckermani compared to other species of *Leishmania*, also reported by Paperna et al. (2001), is that it is only observed infecting erythrocytes instead of also infecting leucocytes, such as *L. (S.) platycephala*. An exception is one instance where a single infection of a 'monocytic leucocyte' in the spleen was reported by Paperna et al. (2001), however we are unable make a comparison in this study, as only the peripheral blood was screened and the gecko host specimens within this study were not dissected. In the present study, amastigotes were infrequently observed extracellularly, apparently due to damaged erythrocytes, even throughout the long-term monitoring of the infected captive host.

While Paperna et al. (2001) counted up to 50 *L. (S.)* zuckermani amastigotes in a single erythrocyte, significantly fewer parasites of *L. (S.)* zuckermani were observed in the present study with the highest number observed being eight amastigotes. However, it should be noted

that a specimen with relatively high parasitaemia was described in the former study. In comparison, L. (*Sauroleishmania*) *platycephala* was reported to have up to 16–24 parasites within erythroid cells (Telford, 2009).

Furthermore, additional evidence determined by Paperna et al. (2001) for the basis of the description of *L. (S.)* zuckermani as a distinct species was due to the specific host (Chondrodactylus turneri) and geographic location (South Africa) being significantly different from known species of *Leishmania*. Therefore, additional supporting information for diagnosis of the species described in the present study as *L. (S.)* zuckermani is that it was isolated from a sister species (*C. bibronii*) to the type host, from the same geographic region as the type locality, South Africa. The type host, Chondrodactylus turneri (syn. Pachydactylus turneri), from which Paperna et al. (2001) originally described L. zuckernmani, was only recently taxonomically split from *C. bibronii* in 2021 (Heinz et al., 2021).

The species-level identification of *L. (S.)* zuckermani is further supported by the novel molecular data presented in this study. In both ITS and 18S phylogenetic analyses, the sequences form a distinct monophyletic clade within the broader *Leishmania* cluster. This unique placement, not clustering with any other available sequences, indicates that they belong to a *Sauroleishmania* species previously lacking molecular data and, when considered alongside the data, supports the identification as *L. (S.)* zuckermani. Both genetic markers show that this species is closely related to, yet distinct from, other species within the *Sauroleishmania* clade, namely, *L. (S.) adleri*, *L. (S.)* gymnodactyli, *L. (S.) hoogstraali* and *L. (S.) tarentolae*, all of which are known to infect gecko hosts (McMillan, 1965; Espinosa et al., 2018; Mendoza-Roldan et al., 2022b).

For these reasons, and based on the morphological evidence as well as the close correspondence of the present material with the type locality and type host described by Paperna et al. (2001), we hereby describe and classify this species as Leishmanania (*Sauroleishmania*) zuckermani, thereby confirming its validity as a distinct taxonomic species. Furthermore, we provide the first molecular data for this species, which will facilitate future diagnosis and comparative analyses.

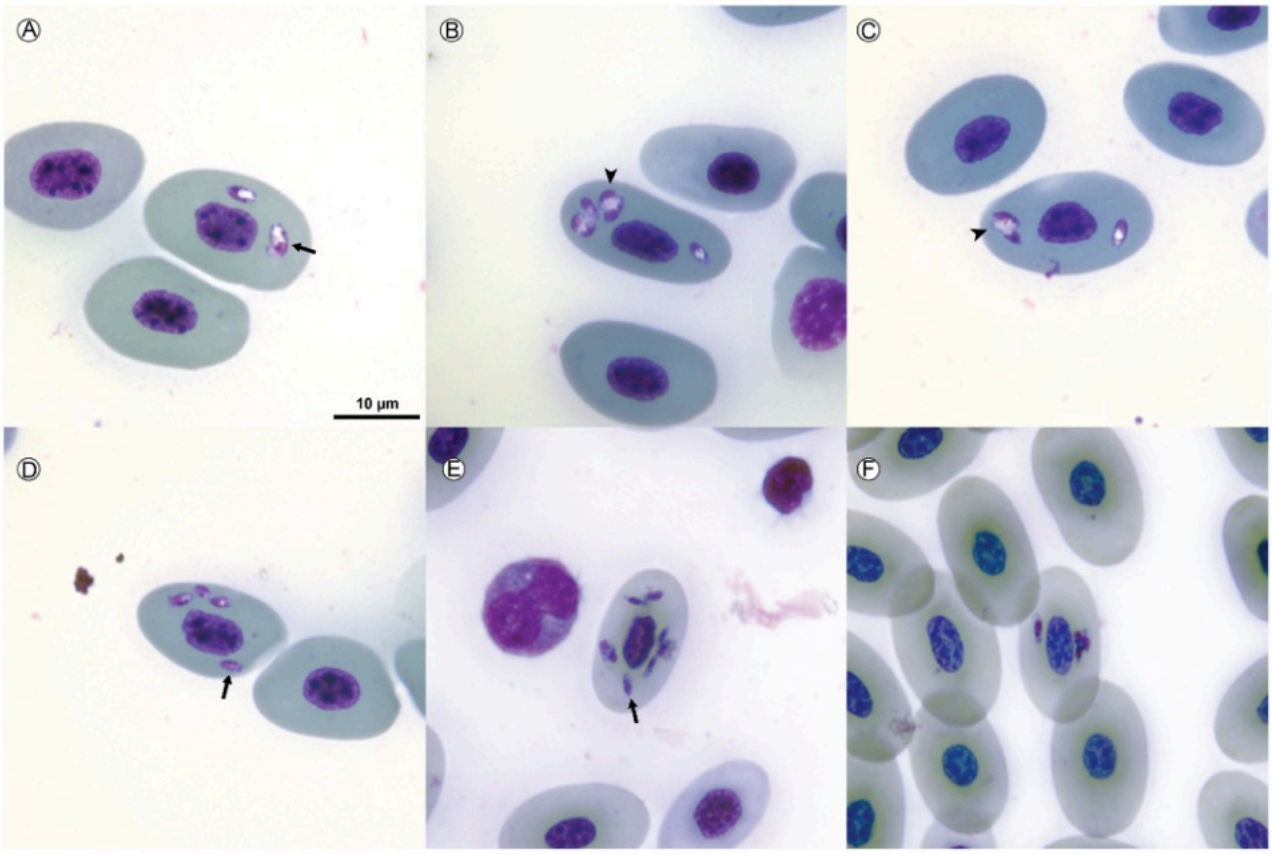

Fig. 2. Photoplate of *Leishmania* (*Sauroleishmania*) zuckermani amastigotes (A–F) ex. *Chondrodactylus bibronii* from South Africa. Arrows indicate amastigotes and arrowheads indicate aggregates.

Phlebotomine sand flies collection and Leishmania screening

A total of 62 phlebotomine sand flies (30 males and 32 females) were collected from 3/8 investigated sites. The observation of key characters in a subset of better conserved specimens, including recumbent setae and small sockets on the abdominal tergites in a subset of specimens, is highly suggestive of the genus Sergentomyia (Dantas-Torres et al., 2014). However, none of the female sand flies tested positive for the presence of *Leishmania* DNA after the ITS-1-targeted PCR molecular screening.

Phylogenetic placement of Leishmania sequences

Both 18S rRNA gene and ITS-1 sequences obtained from the two infected *C. bibronii* were identical between the two blood samples of origin. After a nBLAST search of the 18S sequence, the 137 best hits belonged to different taxa included in the Leishmaniinae subfamily, as well as to other trypanosomatids. All of which showed a query cover of 100 % and percentage identity

ranging from 97.33 % to 98.11 %. The first 33 best hits, according to the BLAST Max Score, belonged to various species of *Leishmania*. Their percentage of identity ranged from 97.52 % to 99.74 %. The same value of identity percentage to the query sequence was shared by different *Leishmania* species. In the bootstrap consensus tree of the 18S ML phylogeny [Fig. 3], the sequences generated in the present research cluster in a monophyletic *Sauroleishmania* clade formed by L. *adleri*, L. *tarentolae*, L. gymnodactyli and L. hoogstrali sequences (bootstrap support value: 0.46 %). This clade is sister to a clade of *Leishmania* species belonging to the *Leishmania* subgenus (bootstrap support: 0.51 %). Within the *Sauroleishmania* clade, the generated gecko sequences form a monophyletic group with those of L. hoogstraali. The L. *adleri* and L. gymnodactyli sequences are sister to a monophyletic clade comprising *L. tarentolae* sequences (bootstrap support value: 0.73 %) and are sister to the clade of the gecko plus L. *hoogstraali* sequences. The nBLAST search of the ITS-1 sequence revealed 140 hits belonging only to the genus of *Leishmania*, with query cover ranging from 95 % to 100 % and percentages of identity ranging from a minimum of 92.45 % to a maximum of 94.86 %. The vast majority of the sequences originated from reptiles (lizards and snakes) and humans from China (Chen et al., 2019; Yang et al., 2010; Zhang et al., 2016), and sand flies of the species *S. minuta* from Spain and Portugal (Gonz ález et al., 2020; Maia et al., 2015). In the bootstrap consensus tree of the ITS-1 ML phylogeny [Fig. 4], the sequences generated in the present research cluster in a monophyletic *Sauroleishmania* clade formed by L. *adleri*, L. *tarentolae*, and *Leishmania* sp. sequences (bootstrap support value: 0.56 %). This clade is sister to a *Leishmania* clade, which comprises species of the subgenus *Leishmania* (bootstrap support value: 0.86 %). Inside this *Sauroleishmania* clade, they constitute a monophyletic clade sister to L. *adleri* (bootstrap support value: 0.98 %). The clade which comprises the sequences generated in the present research and the L. *adleri* sequence is monophyletic (bootstrap support value: 0.56 %) and sister to a monophyletic clade composed of the *L. tarentolae* and *Leishmania* sp. sequences (bootstrap support value: 0.68 %).

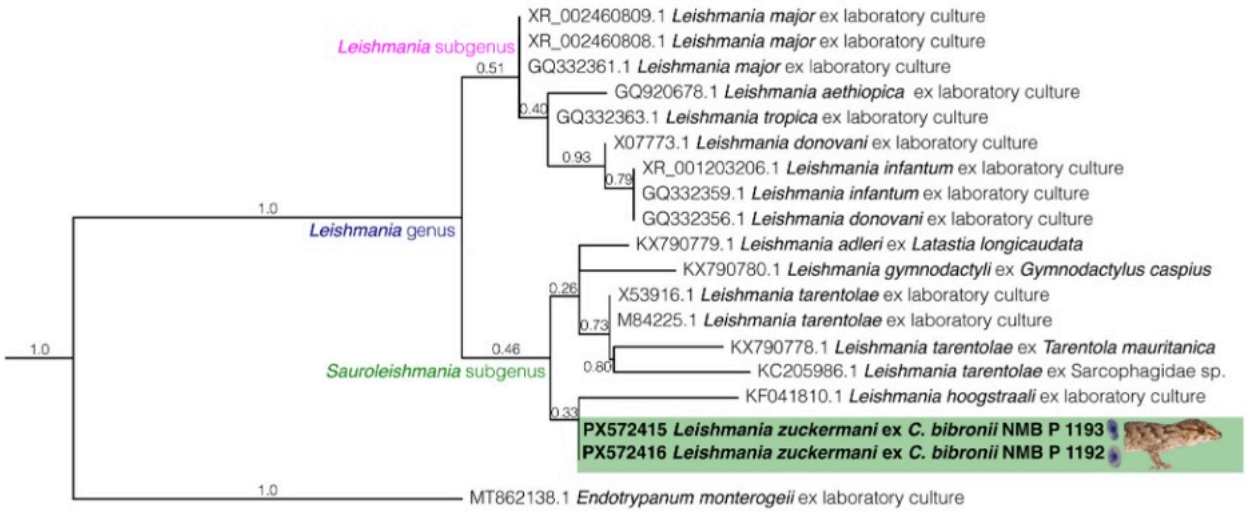

Fig. 3. Maximum Likelihood phylogeny of African *Leishmania* 18S rDNA marker.

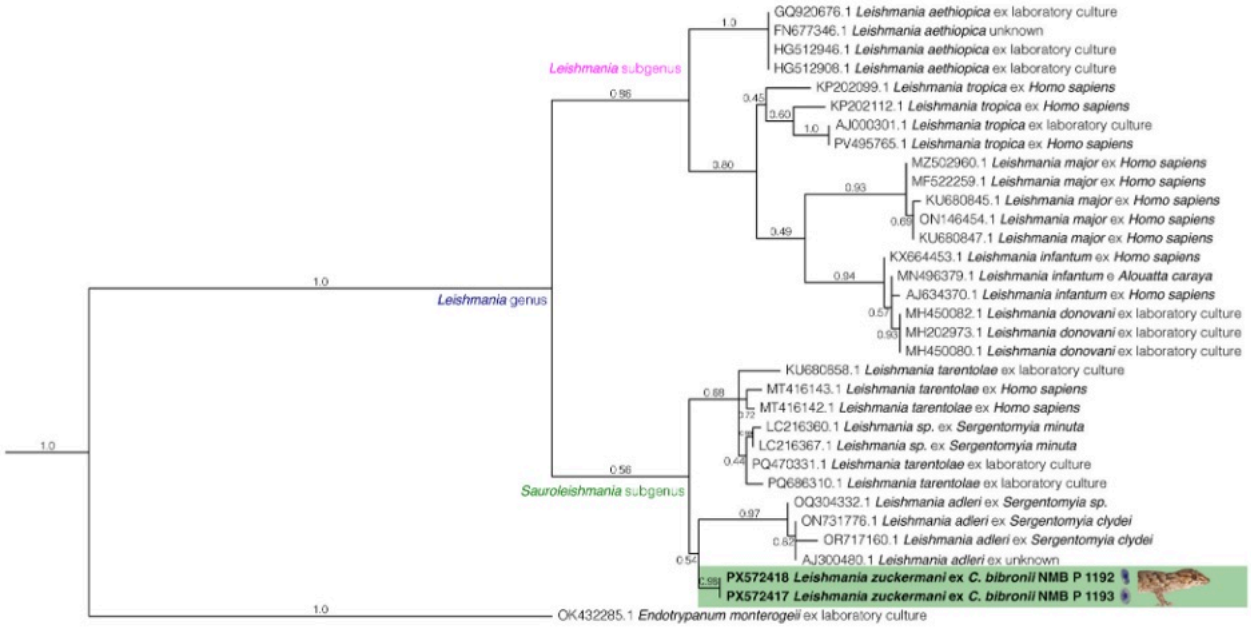

Fig. 4. Maximum Likelihood phylogeny of African *Leishmania* ITS1 marker.

Haemoculturing

Cultures were maintained for up to 3 weeks at 26 ◦C under aerated conditions and monitored twice a week. During this period, no evidence of *Leishmania* was observed in any of the field-collected vials, nor in the molecular screening of the corresponding blood samples from 2025. Similarly, attempts to isolate *Leishmania* from the previously infected gecko (RE231006C1) were unsuccessful, as the isolation was performed after 490 days in captivity, at which point a *Sauroleishmania* infection was no longer detectable. Due to the circumstances of the remote field excursion, isolated gecko blood was immediately transferred into the pre-prepared culture

medium in situ, and returned to the laboratory for incubation and further analysis. As such, the authors were not able to confirm the negative microscopic screenings with molecular screening immediately on site, and still attempted culturing in the event that the parasitaemia was extremely low, as has been described in the literature where successful culturing was performed where infections were not visible in the blood to other authors (Ranque, 1973).

Discussion

According to Telford (2009), the morphology of *Sauroleishmania* amastigotes (not referring to ultrastructural characteristics) in stained blood smears of the host is considered "nearly useless" for species differentiation, relative to other Kinetoplastid flagellate parasites of reptiles, such as trypanosomes. Paperna et al. (2001) stated species of *Leishmania* could not be differentiated by comparing the ultrastructural morphology of the amastigotes, however Lewis (1975) was able to demonstrate differences between *Leishmania* and *Sauroleishmania* spp. based on the microtubule morphometrics of promastigote forms (Telford, 2009).

Microscopic screening of prepared blood smears has been shown to prove challenging in determining positive infections in cases of low parasitaemia, as demonstrated by previous studies (Belova, 1971; Ranque, 1973; Telford, 2009). For this reason, Belova (1971) and Telford (1995) suggest that culture isolation of specimens could be the most reliable technique of demonstrating discovering positive *Sauroleishmania* infections. However, this was prior to the adoption of molecular screening techniques currently in use. As none of the geckoes that were used for the culturing attempts tested positive for *Leishmania* by microscopic or molecular screening, it was unsurprising that no parasites could be propagated in culture. Alongside the blood isolated from specimens collected in the field, culturing was also attempted with blood isolated from the captive gecko specimen. Despite being previously infected up until 75 days prior, at the time of attempted culturing, a positive infection was no longer observed in the captive specimen with microscopic screening. While culturing was unsuccessful in the present study, a combination of microscopy, molecular screening, and culture isolation could prove valuable in diagnosing and characterising infections.

Species of *Sauroleishmania* infecting other reptile hosts are typically reported to infect both erythrocytes and leucocytes, throughout the duration of the infection life cycle (Telford, 1995, 2009). However, in the study of Paperna et al. (2001) and in the present study, this was not observed. When blood smears were examined microscopically, intracellular parasites were

only found within the host erythrocytes. The absence of infected leucocytes throughout the developmental cycle in the reptile host would appear to be a unique characteristic only currently known from this species, *L. (S.)* zuckermani.

Additionally, after 415 days, the infection was no longer visible in the peripheral blood of the specimen in the present study. Of course, it is not known, how long the gecko host had been infected prior to being captured and screened. Telford (2009) reported a latent infection phase of roughly 36 days, whereby a gecko host specimen appeared negative with microscopic screening. After 36 days, the positive infection was first observed in the blood of the specimen, which gradually developed over the subsequent 37 days. Forty-one days after the initial positive infection observation's date, Telford describes the infection reaching extremely low parasitaemia, not being able to detect any parasite cells until day 96, whereafter no more parasites were seen until the conclusion of the screening experiment at 150 days total. Although, it should be noted that in the current study, the parasitaemia was a subjective, relative estimation, as the researchers were not able to significantly quantify this reliably due to variations in blood smear consistency, thickness, surface area. Furthermore, low numbers of parasites resulted in cell counts being impractical and prone to detection bias.

It is not known how long or at what stage of infection the infected gecko specimens were in the study by Telford (2009) or in the present study. Despite *L. (S.)* zuckermani not displaying the developmental cycle within the vertebrate host of *L. (S.) platycephala*, it would not be unreasonable to assume the specimen in the current study had been infected for a longer duration at the point of capture compared to the specimen studied by Telford. Because in the present study, the gecko host was already displaying blood cells infected with amastigotes, whereas the host in the study of Telford only showed the onset of an infection 36 days after capture, when amastigotes were first observed in the blood. Thus, it would appear that Telford (2009) collected the specimen after having been relatively recently infected or while still at a relatively early developmental stage of infection. Subsequently, it could be reasonable to suspect that the parasite *L. (S.)* zuckermani could have been infecting the gecko host in the current study for an extended time prior to capture, although unknown how long. Experimental life cycle and transmission studies would aid in resolving this uncertainty and elucidating these exact life cycle details, by controlling the point in time of parasite infection and being able to regularly monitor the infection development thereafter.

Regardless, the infection duration of *L. (S.)* zuckermani was significantly longer than *L. (S.) platycephala*, being 415 and 96 days, respectively, from the patency of infection until the infection was no longer detected in the blood. This highlights that *L. (S.)* zuckermani has a notable chronic infection period and has further implications about the infectivity period and effect on the host.

While the *Sauroleishmania* infection in this study was only observed in the peripheral blood, it is not known if the parasite infected other cells or tissue. The present study and that of Paperna et al. (2001) only found the parasite amastigote infection in the peripheral blood (infecting erythrocytes), except for single amastigotes infecting leucocytes isolated from the spleen reported by Paperna et al. (2001). No parasites were reported by Paperna et al. when examining the liver, kidney, bone marrow, or other tissue during the dissection of an infected specimen with high parasitaemia. No cultures were attempted during the study of Paperna et al. While culturing of flagellates in this study was unsuccessful, if a successful culture were to be established in future, it would be of great value for further experiments.

Definitive species-level identification within the Phlebotominae subfamily typically requires detailed examination of characters such as the cibarial armature, spermathecae in females, and male genitalia under high-magnification light microscopy (Cecílio et al., 2022). Molecular barcoding analyses are also useful. However, the definitive taxonomic identification of these sand fly specimens was beyond the scope of the present study, which primarily aimed to document the presence of potential vectors and confirm the feasibility of local transmission cycles for *Sauroleishmania*. Although Sergentomyia is the genus most frequently associated with *Sauroleishmania* transmission (Bandi et al., 2023; Lozano Sardaneta et al., 2018; Mendoza-Roldan et al., 2022a), members of the genus Phlebotomus, which, along with Sergentomyia, represent the only two genera of sand flies present in Africa, have also been implicated in the transmission of reptilian *Leishmania* species (Alvaro et al., 2025; Mendoza-Roldan et al., 2022a; Ticha et al., 2021). Thus, the detection of either genus may be consistent with the observed pattern of infection in local geckos. The confirmed presence of sand flies in Bankfontein is highly suggestive of a potential circulation of *Sauroleishmania* parasites. Nevertheless, none of the screened female sand flies tested positive for *Leishmania* DNA. This absence of infection in the vectors could be attributed to a generally low prevalence of the parasite in the gecko population, or its focal distribution in microhabitats not captured by our sampling. Furthermore, the apparent discrepancy between the sites where infected geckos

were recorded (in 2023) and the absence of sand flies in those same locations during our 2025 survey may be explained by a significant temporal lag between host and vector sampling. Despite the low bootstrap support values for intracladistic resolution of relationships, the clades of the phylogenies generated within this study are consistent with accepted phylogenies in the literature for the subgenera of *Leishmania* and *Sauroleishmania* (Kostygov et al., 2021; Mendoza-Roldan et al., 2024).

It is not known if the infection prevalence is seasonal or varies due to other factors. Both infected specimens were caught at different seasons of the year. The first infected specimen (RE231006C1) was caught during the early rain season of the region (October 6, 2023). Upon returning the subsequent year, the second infected specimen (RE240309D1) was collected at the end of the rain season (March 9, 2024). Despite even more focussed efforts and increased samples (from the same and additional localities), the third visit during March of 2025 found no infected specimens. It is not known whether this is possibly related to host or vector activity. A coinfection with species of Haemocystidium Castellani and Willey, 1904 was observed in one *Sauroleishmania* infected host. Coinfections with species of Haemocystidium and Hepatozoon Miller, 1908 were also reported in the study of Paperna et al. (2001).

It was observed during sampling that some rocky areas where both sand flies and geckoes were found were shared habitat with the Rock Hyrax, Procavia capensis (Pallas, 1766), a known reservoir host of *Leishmania* sp. (Talmi-Frank et al., 2010). However, none of these gecko or sand fly specimens were found to be positive for any species of *Leishmania*. The potential of the reptile hosts to act as reservoir hosts for *Leishmania* parasites that could infect mammalian hosts requires further investigation.

Conclusion

In the present study, a supplementary description of *L. (S.)* zuckermani is provided, with the first associated molecular data for this species. An increase of the sampling size and additional sampling sites of geckoes and sand flies, would be valuable in many aspects in gaining more insight into this species. While the prevalence of *L. (S.)* zuckermani was low within this study, the long-term monitoring of an infected individual showed the ability of a chronic infection remaining for multiple years. Despite the observed co-presence of both the gecko host and sand flies, the absence of infected sand flies, does not rule them out as suspected potential vectors, given the low parasite prevalence. If in future studies, parasites are able to be cultured,

this could be further investigated with xenodiagnoses and life cycle studies. While culturing of flagellates in this study was unsuccessful, culturing of samples (initially presenting negative with microscopic screening) has been shown by past studies to be valuable in revealing infections with low parasitaemia. Additionally, molecular analyses proved to be an effective technique for both screening and species characterisation. As the full life cycle, infection duration, transmission route, and distribution of this species is not currently fully understood, the effect of these reptiles to act as reservoir hosts and zoonotic potential needs to be further explored.

CRediT authorship contribution statement

Bernard J. Jordaan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. Alessandro Alvaro: Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. Giulia Cattaneo: Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. Monique Barnard: Investigation, Resources, Writing – original draft, Writing – review & editing. Sara Epis: Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. Edward C. Netherlands: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest declaration

All authors declare no conflict of interest regarding this manuscript.

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8 Discussion and Conclusion

L. tarentolae has long been considered a valuable expression system for the production of heterologous proteins/antigens. Its post-translational modifications are similar to those in mammals. The parasite also grows rapidly and requires minimal biosafety concern (Basile and Peticca 2009a; de Oliveira et al. 2019). Compared to conventional cell factories - such as bacteria (which lack complex glycosylation), yeast (which often introduce hyper-mannosylated glycan structures), and mammalian cells (which are expensive and require stringent biosafety measures) - *L. tarentolae* offers a good alternative, combining scalability, correct protein folding, and cost-effective cultivation (Lai et al. 2019).

Traditional growth media, however, contained animal-derived components such as porcine hemin, FBS, or FCS. This has limited biomedical applications, as regulatory standards require the elimination of animal-derived components to ensure safety, reproducibility, and to minimize the risk of adventitious agents (Whitford et al. 2018; Brunner et al. 2010; Sidana et al. 2017).

In this context, the development of a fully chemically defined medium became crucial for the clinical translation of *L. tarentolae*–based platforms.
In the first paper of this thesis (Cattaneo et al., 2024), a chemically defined medium was developed based on Schneider's Drosophila medium supplemented with horseradish peroxidase (HRP) as a substitute for porcine hemin. *L. tarentolae* adapted to this medium maintained robust growth and efficient recombinant protein expression. Validation using clones expressing the SARS-CoV-2 RBD antigen confirmed that the medium supports both parasite viability and high-quality protein production. These results overcame previous regulatory limitations and enhanced *L. tarentolae* suitability as a scalable eukaryotic expression system.

Building on this, the second paper present in this thesis (Epis et al., 2022) demonstrates the efficiency of *L. tarentolae* as a scaffold for both recombinant antigen production and delivery, which can be used for vaccination purposes. *L. tarentolae* platform was exploited for the production and delivery of SARS-CoV-2 antigens, assessing its potential as an antigen-delivery vehicle for mucosal vaccination in murine model. As a matter of fact, rectal delivery of the antigens included in the LeCoVax-2 vaccine elicited both IgG and neutralizing antibody responses, demonstrating the suitability of *L. tarentolae* as a live mucosal vaccine vector.

Further immunological investigations (Varotto et al., 2023) were conducted to evaluate the potential of *L. tarentolae* to elicit a specific immune response following rectal administration. The results showed a Th1-biased response, characterized by a predominance of IgG2 antibodies, which is advantageous for protection against *Leishmania* infection. This approach also exploits intrinsic advantages of the enteral vaccination. First, mucosal vaccination can stimulate the production of secretory IgA at the site of infection, while taking advantage of the immune-modulatory functions of the gut-associated lymphoid tissue (GALT), which continuously interacts with the microbiota and dietary antigens to maintain immune homeostasis (Hosomi and Kunisawa 2020; Luciani et al. 2022). In addition, the enteral vaccination against *Leishmania* was investigated only in one previous study (Pinto et al. 2003) that exploits the oral delivery of *L. amazonensys* lysate in mice, obtaining a Th1-biased immune polarization. These results ,together with the ones obtained on mice by Varotto et al. XXX, support the suitability of mucosal vaccination strategies for the development of vaccines against *Leishmania* spp.

In parallel, in the fourth publication included in this thesis (Louzada-Flores et al. 2023), *L. tarentolae* biosafety was investigated. Previous studies report that its DNA has been detected in humans and dogs, raising questions about host range (Pombi et al. 2020; Iatta et al. 2021; Mendoza-Roldan et al. 2022). Moreover, *L. tarentolae* vector (i.e. *Sergentomyia minuta*) has been shown to feed also on humans and dogs (Abbate et al. 2020). These findings suggest that *L. tarentolae* may have a broader host range than previously thought (Maia and Depaquit 2016). The co-existence of *L. infantum* and *L. tarentolae* in the same geographical area raises questions about the possible capability of *L. tarentolae* to adapt and persist in non conventional mammalian hosts (Mendoza-Roldan, Votýpka, et al. 2022).

Therefore, our study investigated the ability of *L. tarentolae* to infect, survive and persist in monocyte-derived canine macrophages.

Recent studies have already demonstrated the ability of *L. tarentolae* to be internalized and to develop intracellularly in both murine and human macrophages and dendritic cells, higliting its potential immunogenicity (Breton et al. 2005; Bandi et al. 2023; Taylor et al. 2010). However, there was no clear evidence of *L. tarentolae* persistence in canine macrophages. Thus, this

study highlighted the ability of *L. tarentolae* to persist intracellularly in canine macrophages without inducing cytotoxic effects, supporting its biological safety.

These findings supports a unified framework: from the optimization of the parasite as a cellular production platform, to the characterization of its host–pathogen interactions, and to the application of *L. tarentolae* as a mucosal vaccine delivery system. Together, these results reinforce the rationale for the biomedical exploitation of *L. tarentolae*.

L. tarentolae epidemiological scenario was investigated in the last two publications included in the thesis (Alvaro et al. 2025). As mentioned above, the ecology of *L. tarentolae* remains largely unexplored and recent evidences suggest that the epidemiological scenario could be more complex than previously assumed.

This parasite has been detected in sympatry with the pathogenic *L. infantum* in area in which leihsmaniasis is endemic, and co-infections between these two species have also been reported (Mendoza et al. 2021). *L. tarentolae* DNA has been detected not only in its natural vector (*S. minuta*), but also in other sand fly species such as *Phlebotomus perniciosus* (a proven vector of *L. infantum*) (Ticha et al. 2021). Although *S. minuta* shows a preference for reptiles, it has been shown to occasionally feed on mammals, including humans (Abbate et al. 2020; Gonzales et al. 2020). These findings indicate that the potential host range of *L. tarentolae* may be broader than previously thought.

Reptiles may also play a broader role in the epidemiology of *Leishmania* parasites. Indeed, pathogenic *Leishmania* DNA, such as *L. infantum* and *L. donovani*, has been detected in several reptile species, suggesting their possible contribution to parasite maintenance in endemic areas (Pombi et al. 2020).

Given the limited information available from northern Italy on the prevalence of *Leishmania* spp. in the common wall lizard (*Podarcis muralis*) and in the sand fly *Sergentomyia minuta*, the publication number five and six (see chapter 7.1.5 and 7.1.6) focused on assessing sand fly community composition and detecting *Leishmania* spp. in both reptile and sand fly hosts.

Traditional diagnostic approaches, including microscopy and conventional PCR, have limited sensitivity, especially when parasite DNA is present at low concentrations. To overcome this, droplet digital PCR (ddPCR) offers highly accurate and sensitive detection. In this context, this technique has emerged as a powerful method for achieving highly accurate and sensitive

results. By partitioning the DNA sample into thousands of individual droplets, ddPCR enables absolute quantification of target molecules and overcomes many of the limitations associated with conventional PCR techniques. To date, only three ddPCR assays for *Leishmania* detection have been developed: one designed for the detection of *L. braziliensis* in human clinical samples; a second for the quantification of *L. infantum* in dogs; and a third validated on multiple *Leishmania* species (Ramírez et al. 2019; Pereira et al. 2023). However, no ddPCR assays for *Leishmania* detection in sand fly vectors have been developed to date. In the fifth publication included in this thesis (Alvaro et al. 2025), a novel ddPCR assay targeting kinetoplast minicircle DNA (kDNA) was developed for the simultaneous and sensitive detection of *L. tarentolae* and *L. infantum*. The assay was validated using *L. tarentolae* and *L. infantum* laboratory strains, negative dog blood samples experimentally spiked with cultured parasites, and field-collected sand flies. This method demonstrated high sensitivity for the detection of low parasite loads, achieving a limit of detection of 0.2 cells/µL of dog blood for both *Leishmania* species, while exhibiting minimal cross-reactivity.

This method was subsequently employed to investigate the presence of *Leishmania* spp. synanthropic reptiles and sand flies samples collected in northern Italy, providing new insights into the epidemiological distribution of *L. tarentolae*.

Sand flies and common wall lizards (*Podarcis muralis*) were thus collected in three urban and nine peri-urban sites of the Bergamo district to investigate *Leishmania* spp. presence.

In the peri-urban sites, three sand fly species were identified: *P. perniciosus*, *P. neglectus*, and *S. minuta*. Conventional PCR screening revealed the presence of *Leishmania* DNA in both sand flies and lizard hosts, including blood and fecal samples. DNA sequencing and phylogenetic analyses of these samples revealed that the northern Italian sequences belong to the subgenus *Sauroleishmania*, specifically to the species *L. tarentolae*, reaching a prevalence of 22,2 % in sand flies and 80% in lizard blood.

Digital droplet PCR was subsequently applied to the tested samples, confirming strong circulation of *L. tarentolae*, with a prevalence of 91.9% in sand flies and 97.14% in lizard blood samples. In contrast, *L. infantum* DNA was detected at a low prevalence - 9.46% in sand flies and 8.57% in lizard blood samples - highlighting the assay's higher sensitivity compared to conventional PCR.

Finally, the host range of *Sauroleishmania* was investigated in the last two articles (see Chapters 7.2.1 and 7.2.2), focusing on fire salamanders in northern Italy and gecko species in

South Africa. These studies, in which the latter was supported by data collected during a three-month research period abroad at the University of Free State (Bloemfontein, South Africa), provide new insights into the ecology and host associations of this subgenus. These parasites are traditionally considered to be exclusively associated with reptiles; however, the findings of these studies suggest a broader ecological niche, indicating that synanthropic and widely distributed reptile species may play a significant role in the maintenance and distribution of *Sauroleishmania* in both endemic and non-endemic areas. These findings highlight the need for further research on non-traditional hosts to fully understand *Sauroleishmania* transmission dynamics and their potential impact.

In conclusion, the studies included in this thesis provide a comprehensive perspective on both the biomedical applications and the epidemiology of the protozoan parasite *Leishmania tarentolae*.

This work highlights the optimization of *L. tarentolae* as a safe and scalable eukaryotic expression system, including the development of a fully chemically defined medium, enabling efficient recombinant protein production in compliance with regulatory standards. Furthermore, its immunogenicity and biological safety were demonstrated through in vitro investigations. In addition, *L. tarentolae* proved to be an effective mucosal vaccination platform, eliciting a Th1-biased immune response in murine models and underscoring its potential in enteral immunization strategies.

In parallel, epidemiological investigations of *L. tarentolae* revealed an expanded host range and its coexistence with the pathogenic *L. infantum* in the northern Italy. Phylogenetic analyses provided the first molecular evidence of an autochthonous *L. tarentolae* cycle in this area. Moreover, complementary studies on non-traditional hosts, including fire salamanders and South African geckos, contributed to a broader understanding of *Sauroleishmania* host associations and ecological distribution, providing evidence that the host range of these parasites extends beyond classical reptile species. These findings emphasize the ecological complexity of *Sauroleishmania* transmission and highlight potential implications for parasite maintenance and surveillance in both endemic and non-endemic areas.

Thus, these studies demonstrate the versatility of *L. tarentolae* as a safe, scalable expression system, a mucosal vaccine platform, and a subject for epidemiological investigations, highlighting its biomedical and ecological relevance.

9 Other publications of parasitological and immunological interest

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The non-pathogenic protozoon *Leishmania tarentolae* interferes with the activation of NLRP3 inflammasome in human cells: new perspectives in the control of inflammation

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Background: Innate immune responses against infectious agents can act as triggers of inflammatory diseases. On the other hand, various pathogens have developed mechanisms for the evasion of the immune response, based on an inhibition of innate immunity and inflammatory responses. Inflammatory diseases could thus be controlled through the administration of pathogens or pathogen-derived molecules, capable of interfering with the mechanisms at the basis of inflammation. In this framework, the NLRP3 inflammasome is an important component in innate antimicrobial responses and a major player in the inflammatory disease. Parasites of the genus *Leishmania* are master manipulators of innate immune mechanisms, and different species have been shown to inhibit inflammasome formation. However, the exploitation of pathogenic *Leishmania* species as blockers of NLRP3-based inflammatory diseases poses safety concerns.

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Spauligodon orobicus sp. nov. (Oxyurida: Pharyngodonidae) a parasite infecting the common wall lizard, *Podarcis muralis* (Laurenti, 1768) in northern Italy

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Abstract

Parasitic nematodes of the oxyurid family Pharyngodonidae are commonly found across reptile orders, with species of the genus *Spauligodon* Skrjabin, Schikhobalova & Lagodovskaja, 1960, being among the most common ones. However, scant information exists regarding the prevalence of *Spauligodon* nematodes in Italian reptile populations. Although two *Spauligodon* species have been reported from southern Italy, the presence of these worms in the rest of the country remains unknown. In this research, we describe *Spauligodon orobicus* sp. nov. from an Italian common wall lizard *Podarcis muralis* (Laurenti, 1768) of northern Italy. The new species is distinct from other known species both at the morphological and molecular level. The study increases the knowledge on the biodiversity of *Spauligodon* nematodes and in general on the biodiversity of Italy, and adds northern Italy to the geographical range of this nematode genus.

http://zoobank.org/urn:lsid:zoobank.org:act:29DB5E90-9CB5-46A3-8471-FEE0395FA29A

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Interactions between the pathogenic fungus *Cryptococcus neoformans* and ants

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ABSTRACT

Despite the growing number of environmental surveys aimed to understand the ecology of the fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*, little is known about their relationships with arthropods. In the present study we collected a large number of samples from trees and arthropods living on them to determine the occurrence of *Cryptococcus* in arthropods, to understand if they could represent a vehicle for dispersion in the environment, and finally to investigate how they might interact with the fungus. Samples were collected from seven different geographical areas of the world: northwestern Italy, southeastern Italy, Slovenia, Kosovo, Greece, Turkey, and Colombia. A total of 1396 trees were examined and 11,805 samples were collected, including 7492 arthropod samples. Arthropod positive samples, mostly from ants, were found only in northwestern and southeastern Italy, Greece, and Slovenia with an average rate of 0.2%. Thirty-three of positive trees hosted positive arthropods whereas in six of them arthropods resulted negative. In addition, for six trees, positive samples from arthropods were not associated with positive arboreal samples. In vitro experiments showed that ants can transfer cryptococcal yeasts from a contaminated substrate (soil or bark) to a sterile one and that the fungus can survive inside the digestive apparatus of ants. The present study showed that ants are potential vehicles for *C. neoformans* although the frequency of which they enter in contact with the fungus is low. Cryptococcal yeasts can survive within the bodies of ants, but it remains unclear whether the relationship they establish with their host is parasitic, commensal, or symbiotic.

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