Title: Prevalence and Risk Factors of *Plasmodium* Infection in Dromedary Camels (*Camelus dromedarius*) of Sistan and Baluchestan, Iran

Running title: *Plasmodium* Infection in Dromedary Camels of Iran

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Abstract

Introduction: Understanding *Plasmodium* infections in camels is of great importance for animal health and for assessing potential zoonotic transmission risks. Hence, the present study aims to explore the prevalence of *Plasmodium* infection in camels in the province of Sistan and Baluchestan in Iran.

Material and Methods: A cross-sectional study was conducted from November 2022 to July 2024. A total of 270 camels (111 samples in spring, 93 in summer, 41 in autumn, and 25 in winter) were randomly selected from Sistan and Baluchestan province, and 5 ml of jugular vein blood was collected from each. Furthermore, Nested-polymerase chain reaction (PCR) targeting the mitochondrial cytochrome b gene of *Plasmodium* was performed on the same samples using DW2/DW4 primers (primary reaction), followed by NCYBINF/NCYBINR primers (nested reaction). Moreover, risk factors associated with infection, including age, sex, geographic location, season, and environmental factors, were evaluated.

Results: No *Plasmodium*-like parasites or other hemoparasites were observed in any smear. All erythrocytes appeared morphologically normal, with no ring forms, trophozoites, schizonts, or gametocytes detected. Neither the first-round nor the nested amplification yielded any detectable cytochrome b gene of *Plasmodium*.

Conclusion: This study revealed no evidence of *Plasmodium* infection in dromedary camels in Sistan and Baluchestan province, Iran, as neither microscopic examination nor nested PCR targeting the mitochondrial cytochrome b gene detected any parasite DNA or blood-stage forms.

Keywords: Malaria; One health; Polymerase chain reaction; Ruminants; Vector-borne diseases

1. Introduction

Malaria remains one of the most common and devastating parasitic diseases worldwide, causing hundreds of millions of clinical cases and more than 600,000 deaths annually, mainly in sub-Saharan Africa, parts of Asia, and Latin America [1]. Malaria is an important zoonotic disease caused by protozoan parasites of the genus *Plasmodium* (order Apicomplexa: Plasmodiidae) [2-4]. It affects not only humans but also various bird and mammal species, posing significant challenges to public health and veterinary medicine. Despite significant advances in vector control, diagnostics, and antimalarial drugs, the emergence of drug resistance and the persistence of zoonotic reservoirs remain significant obstacles to the global elimination of malaria.

The *Plasmodium* life cycle commences when sporozoites, the parasites, are generated in the insect vector and subsequently infiltrate the bloodstream of the vertebrate host after a bite [5]. Sporozoites introduced into the dermis quickly traverse to the liver and infiltrate hepatocytes, proliferating exponentially in schizogony. The resultant parasites, termed merozoites, are released into the bloodstream and infect erythrocytes. In an erythrocyte, a single merozoite undergoes asexual reproduction, producing between 8 and 64 new merozoites [6]. The newly formed merozoites are released into the bloodstream, and the parasites resume this intraerythrocytic propagation cycle every 24 hours (*P. knowlesi*), 48 hours (*P. falciparum*, *P. ovale*, *P. vivax*), or 72 hours (*P. malariae*) [4, 7, 8]. Merozoites then differentiate into the subsequent developmental stage, the gametocyte, for sexual reproduction. The initiation of gametocyte differentiation (gametocytogenesis) varies by species.

Camel (*Camelus* spp.) farming is important due to expanding milk and meat production, and dromedaries in endemic ecological areas have come into increased contact with human populations [9, 10]. Although camels have traditionally been considered relatively resistant to

Plasmodium infections, sporadic reports of blood parasites in these hosts suggest they may serve as under-recognized reservoirs for malaria parasites. Understanding the diversity and prevalence of *Plasmodium* species in camels is of great importance for animal health and for assessing potential zoonotic transmission risks.

Only five *Plasmodium* species naturally infect humans and cause malaria across extensive regions of the world: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* [4, 11]. The initial four are exclusive to humans, whereas *P. knowlesi* is mostly sustained in macaque monkeys and is responsible for zoonotic malaria prevalent throughout Southeast Asia. The transmission of *Plasmodium* species among vertebrate hosts relies on an insect vector, typically a mosquito [4, 12]. All five *Plasmodium* species responsible for malaria in humans are solely transmitted by anopheline mosquitoes.

Diseases transmitted from camels to humans in Iran include 19 zoonotic diseases (including leptospirosis, listeriosis, plague, brucellosis, campylobacteriosis, Q fever, tuberculosis, pasteurellosis, *Escherichia coli* infections, clostridiosis, rabies, camelpox, salmonellosis, Middle East respiratory syndrome coronavirus, echinococcosis, Crimean-Congo haemorrhagic fever, toxoplasmosis, cryptosporidiosis, and dermatophytosis) [13]. Malaria is not mentioned in this list, indicating a gap in information on this topic.

As a result, the purpose of this research is to explore the prevalence of *Plasmodium* infection in camels in the province of Sistan and Baluchestan in Iran, as well as the risk factors associated with developing the infection, and then compare these findings to the incidence and prevalence of *Plasmodium* infection in camels across the globe.

2. Materials and Methods

2.1. Study Area

The present study was conducted in Sistan and Baluchestan Province, in southeastern Iran. The province is characterized by a predominantly hot and dry climate, with an average annual precipitation of 51 mm, an average temperature of 12–13 °C, and a relative humidity of 70–80% [14]. Molecular experiments were performed in the Faculty of Veterinary Medicine, Zabol University laboratory.

2.2. Sampling and Study Population

A cross-sectional study was conducted from November 2022 to July 2024 (111 samples in spring, 93 in summer, 41 in autumn, and 25 in winter). A total of 270 camels were randomly selected from Pishian, Dashtiari, Chabahar, Mirjaveh, and Zahedan cities, and about 5 ml of jugular vein blood was collected from each. The samples were transported to the laboratory in an ice box and stored in a freezer at -20 °C until further evaluation (Table 1).

Table 1. Sex, Demographic characteristics, seasonal distribution, and geographical origin of the sampled dromedary camels (*Camelus dromedarius*) included in the present study in Sistan and Baluchestan Province, Iran.

Variable	Category	n	%
Sex	Male	111	41.1
	Female	159	58.9
Age group (years)	<5	62	23.0
	5–10	137	50.7
	>10	71	26.3
Season of sampling	Autumn	41	15.1
	Winter	25	9.3
	Spring	111	41.1

	Summer	93	34.5
Geographical location	Pishian		
	Dashtiari	48	17.8
	Chabahar	78	28.9
	Mirjaveh	36	13.3
	Zahedan	108	40
Total		**270**	**100**

2.3. Preparation of Blood Smears and Microscopic Examination

A drop of blood from the camel's ear vein was placed on a clean slide and spread using a candle flame to create a one-cell-thick layer. After fixation with methanol, staining with Giemsa (4 ml Giemsa + 6 ml distilled water) was performed. After 45 minutes of washing, the smears were examined under a 100x lens to confirm or rule out infection with blood parasites.

2.4. Molecular analysis

DNA extraction from whole blood was performed using the Denazist extraction kit according to the manufacturer's protocol. The purity and concentration of the extracted DNA were evaluated with a nanodrop spectrophotometer to prepare it for polymerase chain reaction (PCR). Molecular detection of the *Plasmodium* parasite was performed based on the Nested-PCR technique. In the initial reaction, the general primer pair DW2/DW4 and the nested reaction with specific NCYBINF/NCYBINR were performed to target the mitochondrial cytochrome b gene (Ref). To visualize the PCR products, they were loaded into a 2% agarose gel in 1× TAE buffer containing ethidium bromide at a 0.1 µg/mL concentration. Electrophoresis was performed at 100 V for 40 minutes, and the bands were visualized under UV light (Tables 2 and 3).

Table 2. Primer Sequences for *Plasmodium* spp. Mitochondrial Cytochrome b Gene Amplification

Target Gene	Primer	Sequence (5′→3′)	mer	Product Length (bp)	Reference
Plasmodium spp. Cytb	DW2	TAATGCCTAGACGTATTCCTGATTATCCAG	30	1253	[15]
	DW4	TGTTTGCTTGGGAGCTGTAATCATAATGTG	30		
	NCYBINF	TAAGAGAATTATGGAGTGGATGGTG	25	822	
	NCYBINR	CTTGTGGTAATTGACATCCAATCC	24		

Table 3. Thermal Cycling Conditions for Primary and Nested-PCR Amplification of *Plasmodium* Cytb Gene

Reaction	Step	Step Name	Temperature (°C)	Time	Cycles	Notes
Primary PCR	1	Initial Denaturation	95	5 min	1	—
	2a	Denaturation (cycle)	95	30 s	40	—
	2b	Annealing (cycle)	62.5	40 s	40	Primers: DW2 & DW4
	2c	Extension (cycle)	72	60 s	40	Taq polymerase–mediated DNA synthesis

	3	Final Extension	72	10 min	1	Ensures completion of all strands
	4	Hold	4	—	1	Short-term storage in a cycler
	1	Initial Denaturation	95	5 min	1	Template: 1:40 dilution of primary PCR product
	2a	Denaturation (cycle)	95	30 s	40	—
Nested PCR	2b	Annealing (cycle)	60	40 s	40	Primers: NCYBINF & NCYBINR
	2c	Extension (cycle)	72	60 s	40	—
	3	Final Extension	72	10 min	1	—
	4	Hold	4	—	1	—

2.5. Statistical Analysis

SPSS statistical software (version 21) was also used to analyze data. The relationship between the independent variables (age, sex, season, and geographical location) was investigated using the chi-square test. A significance level of $p \leq 0.05$ and a confidence limit of 95% were considered significant.

3. Results

A total of 270 camel blood samples were examined by light microscopy after staining with Giemsa. No *Plasmodium*-like parasites or other hemoparasites were observed in any smear. All erythrocytes appeared morphologically normal, with no ring forms, trophozoites, schizonts, or gametocytes detected. Furthermore, Nested-PCR targeting the mitochondrial cytochrome b gene of *Plasmodium* was performed on the same samples using DW2/DW4 primers (primary reaction), followed by NCYBINF/NCYBINR primers (nested reaction). Neither the first-round nor the nested amplification yielded any detectable cytochrome b gene of *Plasmodium* (Figure 1).

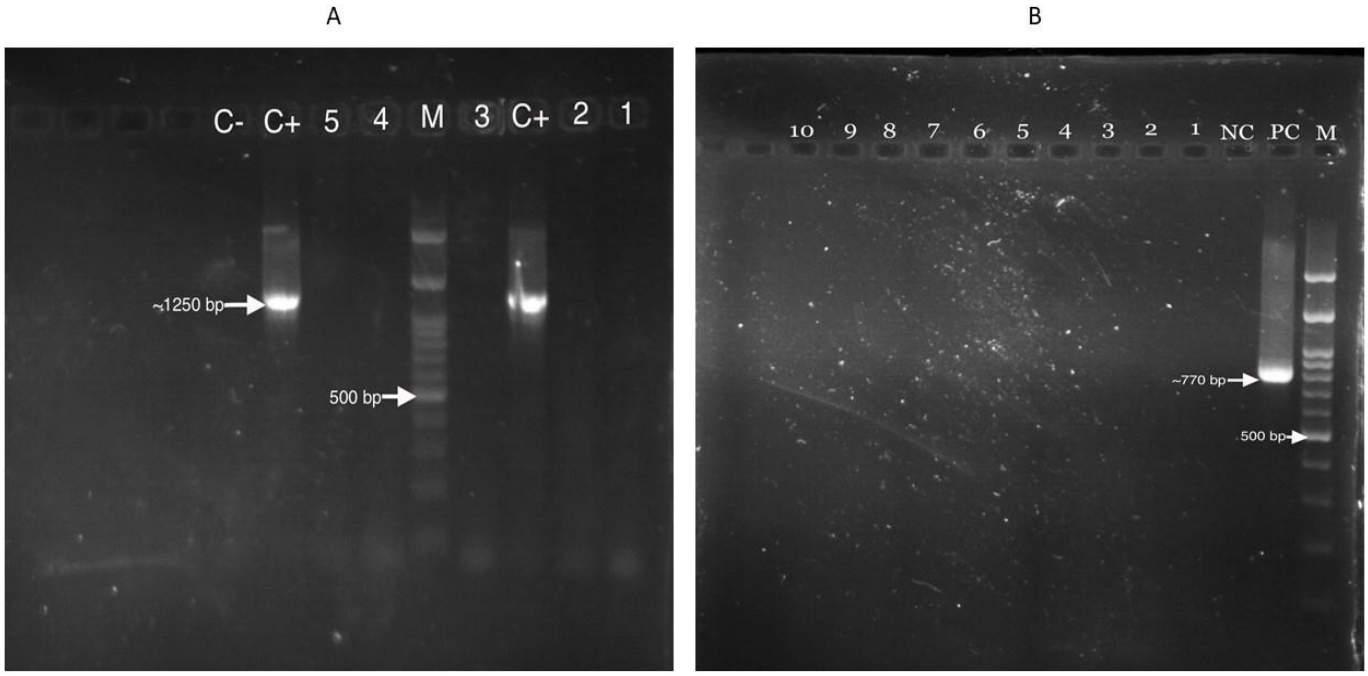

Figure 1.A. Agarose gel electrophoresis of Plasmodium DW2 and DW4 primers for the primary PCR reaction. Lane M: 100-bp DNA ladder; NC: negative control; PC: positive control; lanes showing the primary PCR products obtained from blood samples.

Figure 1.B. Agarose gel electrophoresis of Plasmodium NCYBINF and NCYBINR primers for the nested PCR reaction. Lane M: 100-bp DNA ladder; NC: negative control; PC: positive control; lanes showing the final PCR products from blood samples.

No traces of *Plasmodium* parasites or other hemiparasites were observed in any of the 270 camel blood samples. This study demonstrates that the camel population in this area is free of *Plasmodium* parasites, and the possibility that camels play a role in the malaria transmission cycle appears very weak in light of these findings. Continued epidemiological studies and sampling in different seasons could confirm these results and investigate the environmental and biological factors affecting the absence of *Plasmodium* infection in camels.

4. Discussion

The absence of *Plasmodium* DNA in any of the 270 blood samples analyzed from dromedary camels in Sistan and Baluchestan suggests that these animals are unlikely to play a significant role in the malaria transmission cycle in this region. This finding is consistent with earlier studies from Iran and other parts of the world, where camels have rarely, if ever, been confirmed as competent hosts for *Plasmodium* spp. Notably, despite endemicity in the area and environmental conditions favorable for vector persistence, molecular evidence of infection was not detected, even during the high-transmission season.

Plasmodium can infect a range of even-toed ungulates, including livestock and wild ruminants [15, 16]. Historically identified species include *P. bubalis* (water buffalo malaria) and *P. caprae* (goat malaria). In particular, *P. bubalis* was first described in water buffalo in India, and *P. caprae* in African goats. Other described ungulate malaria parasites include *P. cephalophi* (in duiker

antelope), *P. limnotragi* (marshbuck), *P. traguli* (mouse deer), and *P. odocoilei* (white-tailed deer), but these are wild species. Domestic cattle (*Bos taurus*) have no confirmed *Plasmodium* infections, whereas water buffalo (*Bubalus bubalis*) are susceptible to *P. bubalis*. Small ruminants (sheep and goats) appear to harbor *Plasmodium* at low levels. For instance, a Nigerian survey found blood-stage *Plasmodium* in goats and sheep (the parasite being the most prevalent hemoparasite, 56.3% of infected animals) [17]. Recent molecular surveys across Africa and Asia (Kenya, Sudan, Iran, Myanmar, Thailand) detected a single goat-associated *Plasmodium* lineage in all regions, consistent with *P. caprae* spreading with domestic goats [18]. In summary, ruminant malaria parasites include *P. bubalis* (buffalo) and *P. caprae* (goats), plus related ungulate species; sheep may harbor undetermined *Plasmodium* spp., but no species has been specifically named in cattle or sheep to date [16, 17].

Diagnosing *Plasmodium* spp. can be challenging. Giemsa-stained blood smears (thin films) can reveal intraerythrocytic parasites and hemozoin. In buffalo, trophozoites and gametocytes of *P. bubalis* have been seen by microscopy [16]. Infected erythrocytes may be enlarged with rod-shaped pigment. However, parasitemias in ruminants are usually extremely low (<0.05%) [16], so routine microscopy often misses infections [18, 19]. For instance, an Indonesian study found 7.4% PCR-positive goats, yet all blood smears were negative. A sick buffalo case yielded <0.05% parasitemia by smear [16], underscoring that skilled microscopy may detect malaria in heavily infected animals but not subpatent cases.

Nested PCR targeting parasite genes (e.g., mitochondrial cytochrome b, 18S rRNA) is the gold standard for detection [20]. These assays can detect minute quantities of *Plasmodium* DNA in blood [16, 19]. Studies have successfully used cytb-nested PCR to screen livestock. For instance, 4 of 54 goats in Indonesia were PCR-positive [19], and surveys of 56 Nepalese buffalo

found two positives [21]. Quantitative PCR (qPCR) can even estimate parasitemia (one goat sample showed 0.01% infection by qPCR) [18]. PCR is thus much more sensitive than microscopy for ruminant *Plasmodium* spp.

Significant efforts have been made to eliminate malaria in Iran, but the southern provinces (Sistan and Baluchestan, Hormozgan, and Kerman) still have endemic cases [22-24]. Approximately 77% of the country's malaria cases are recorded in these southern regions, and cases of the disease have recently increased significantly in Sistan and Baluchestan. Environmental conditions and vector populations allow year-round transmission of the disease. However, the role of local camel populations in the malaria parasite transmission cycle has not been comprehensively investigated.

Despite the negative findings of the present study, extremely low-level *Plasmodium* infections cannot be completely ruled out. Conventional diagnostic methods may lack sufficient sensitivity in such conditions. In this regard, nanobiosensor-based diagnostic approaches represent a promising tool for future studies, offering high sensitivity and rapid detection of trace parasitic biomarkers. The application of these technologies could improve surveillance in low-prevalence settings and provide a more accurate understanding of the epidemiology of *Plasmodium* infections in camels and other livestock [25].

Despite the negative results observed in this study, several important future perspectives and challenges remain regarding the surveillance of *Plasmodium* infection in dromedary camels in Iran and globally. First, although nested PCR targeting the mitochondrial cytochrome b gene is a highly sensitive molecular tool, the complete absence of amplification in all 270 samples raises the question of whether true infection is absent or whether very low parasitemia levels, possible chronic carrier states, or latent infections exist that fall below the detection limit of even nested

PCR. Future studies should therefore employ more advanced molecular techniques such as real-time PCR with higher sensitivity, loop-mediated isothermal amplification (LAMP), or next-generation sequencing (NGS) to detect possible cryptic or sub-patent infections. Second, the potential role of camels as accidental or dead-end hosts for zoonotic *Plasmodium* species, particularly *Plasmodium knowlesi* or *Plasmodium vivax*-like parasites, remains largely unexplored in the Middle East. Comparative studies between camels and other domestic ruminants in the same geographical area could clarify host specificity and cross-species transmission dynamics. Third, entomological investigations are critically needed to identify anopheline mosquito species composition, seasonal abundance, and biting behavior in Sistan and Baluchestan province, as the absence of *Plasmodium* in camels may simply reflect a lack of competent vectors rather than inherent host resistance. Fourth, climate change and environmental modifications, including irrigation projects and agricultural expansion, may alter vector distribution and create new transmission foci in arid regions, necessitating periodic reassessment of infection prevalence. Fifth, the lack of standardized sampling protocols, including optimal sample types (whole blood, spleen, or liver tissue), storage conditions, and extraction methods, remains a challenge for comparing results across different studies and countries. Finally, future research should integrate serological assays to detect anti-*Plasmodium* antibodies, which could indicate past exposure even in the absence of detectable parasitemia, thereby providing a more complete picture of historical infection pressure. Addressing these challenges will require multidisciplinary collaborations among veterinarians, parasitologists, entomologists, and public health officials, as well as longitudinal studies covering different ecological zones and seasonal variations over multiple years.

Conclusion

This study revealed no evidence of *Plasmodium* infection in dromedary camels (*Camelus dromedarius*) in Sistan and Baluchestan province, Iran, as neither microscopic examination nor nested PCR targeting the mitochondrial cytochrome b gene detected any parasite DNA or blood-stage forms. These findings suggest that camels in this region are unlikely to serve as a reservoir for *Plasmodium* species, including those with zoonotic potential. The absence of infection may be attributed to ecological factors, such as low vector abundance or reduced vector-host contact, as well as possible host resistance mechanisms. Comparison with global reports indicates that *Plasmodium* prevalence in camels varies considerably by region, and the present study highlights the need for further molecular surveillance across different seasons and geographical areas to better understand the epidemiology of camelid malaria and its potential public health implications.

Declarations and statements

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Author contribution

Conceptualization: [A.S.], …; Methodology: [All Authors], …; Formal analysis and investigation: [All Authors], …; Writing - original draft preparation: [M.K.T., A.S., H.H., M.S.]; Writing - review and editing: [M.K.T., A.S., H.H., M.S.], …; Funding acquisition: [Self-funding], …; Supervision: [A.S.]. All authors checked and approved the final version of the manuscript for publication in the present journal

Conflict of interests:

The authors declare no conflict of interest.

Ethical approval:

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The study procedure was approved by the ethical committee of the Animal Welfare Committee at Zabol University. Informed consent was obtained from all owners, clearly stated in the current study. (Code: IR.UOZ.REC.1404.004).

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Data availability:

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declaration of the use of generative AI

Not used for any purposes

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