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A Comparative Analysis of Loop-Mediated Isothermal Amplification and Polymerase Chain Reaction Assays for the Detection of Tick-Borne Relapsing Fever *Borrelia* in *Borrelia tholozani* Ticks from Northwest Iran

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Abstract

Background & Objectives: The tick *Borrelia tholozani* serves as a principal vector for Relapsing Fever *Borrelia* (RFB), an endemic pathogen in Iran. In this study, we assessed and compared the efficacy of loop-mediated isothermal amplification (LAMP) and polymerase chain reaction (PCR) assays for the detection of *Borrelia* by targeting the *glycerophosphodiester phosphodiesterase* (*glpQ*) gene—a sequence conserved across all RFB species—in *Ornithodoros* (*O*). *tholozani* ticks collected from Northwest Iran.

Materials & Methods: A total of 103 *O. tholozani* ticks were collected from Northwest Iran in 2017. Following DNA extraction, the samples were analyzed using both *glpQ*-LAMP and *glpQ*-PCR assays.

Results: The *glpQ* gene sequence indicative of RFB was identified in 18.44% (19 out of 103) of the ticks when analyzed by *glpQ*-LAMP, whereas the *glpQ*-PCR assay detected RFB DNA in 12.62% (13 out of 103) of the samples.

Conclusion: The *glpQ*-LAMP assay is proposed as a rapid and reliable molecular diagnostic tool for monitoring RFB in ticks from areas endemic for Tick-Borne Relapsing Fever (TBRF).

Keywords: *Borrelia*, *Borrelia tholozani*, *glpQ*, LAMP, PCR

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Introduction

Ticks and tick-borne diseases significantly impact the health of both humans and animals, and they can cause substantial economic losses. Approximately 10% of the 867 known tick species serve as vectors for a diverse array of pathogens affecting mammals and other animals, and they can also inflict direct harm through their biting and feeding activities. The impact of tick-borne diseases on public health and animal production, as well as the imperative for prevention, is considerable (1).

Soft ticks of the genus *Borrelia* (family *Argasidae)* constitute the primary vector for Relapsing Fever *Borrelia* (RFB) (2, 3). Tick-borne relapsing fever (TBRF) is characterized by one or more episodes of fever followed by septicemia and non-specific symptoms such as headache, with these episodes being separated

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by intervals of 4–14 days during which patients experience relative apyrexia (4, 5). Additional non-specific manifestations of TBRF include nausea, vomiting, sweating, abdominal pain, arthralgia, cough, eosinophilia, hematuria, jaundice, petechiae, and scleral congestion. Lyme disease and malaria exhibit clinical features that closely resemble those of TBRF, and therefore should be considered in the differential diagnosis (6). TBRF is an endemic infectious disease across most continents, including Asia, Africa, Europe, and America, with the exception of a few regions in the Southwest Pacific. Iran is recognized as an endemic region for TBRF, and cases are notifiable under the national communicable disease surveillance system (7).

B. persica and *B. microti*, which represent the most prevalent etiological agents of TBRF in Iran, are transmitted by *O. tholozani* and *O. erraticus*, respectively (8). The initial report of *B. persica* was derived from a patient in Ardabil province, Iran, by Dschunkowsky and Luhs in 1913. *Borrelia* ticks, the vectors of *B. persica* and B. microti, are predominantly found in northwestern and southern Iran (6, 9). While *B. latyschewii* and *B. baltazardii* have not been documented in Iran, epidemiological reports strongly implicate *B. persica* and *B. microti* as the principal agents causing human infections (9).

The characterization of RFB is based on the geographic distribution of TBRF patients, along with epidemiological and clinical evidence, the morphological features of RFB, and ultimately the presence of the vector *Ornithodoros*, which is responsible for transmission (10).

A total of 1,415 TBRF cases were recorded throughout Iran during the period 1997–2006. Notably, the provinces of Ardabil (625/1,415 cases), Hamadan (218/1,415), Zanjan (182/1,415), Kurdestan (139/1,415), and Qazvin (66/1,415) rank among the most affected regions (11).

RFB is primarily transmitted through the bite of soft ticks, with the exception of Lyme disease (*B. recurrentis*), which is spread by body lice. Currently, screening of tick vectors for RFB relies on morphological examination to identify *Borrelia* species, in addition to resource-intensive techniques such as PCR-based assays (12). PCR testing requires expensive equipment and highly skilled technicians (13).

Loop-mediated isothermal amplification (LAMP) has proven to be a rapid, highly sensitive and specific, cost-effective, simple, and user-friendly technique compared to conventional PCR-based methods for the detection of various pathogenic agents (14–19). Recently, we reported the successful application of LAMP for the detection of the *glycerophosphodiester phosphodiesterase* (*glpQ*) gene, a conserved sequence among all RFB species (16, 20). Furthermore, we demonstrated the efficacy of LAMP in detecting RFB DNA in dried blood spots (DBSs) from spirochetemic mice and in sera from patients with relapsing fever, in comparison with a *Borrelia*-specific nested PCR. The aim of this study is to evaluate and implement the *glpQ*-LAMP assay for the detection of RFB DNA in soft ticks collected in western Iran, and to compare its performance with that of *glpQ*-PCR.

Materials and Methods

Study Area and Ticks Collection

Surveys were conducted in the Qorveh district of Kurdestan Province in Northwest Iran. The district is a mountainous region with an altitude of approximately 1900 meters above sea level, experiencing very cold winters with heavy snowfall and relatively cool summers. Tick specimens were collected during May and June 2017 from various randomly selected villages in the area. Investigators systematically inspected cracks, crevices, ceilings, and floors of houses to locate ticks, which were subsequently placed in holding tubes. A total of 103 tick specimens, exhibiting morphological features consistent with *O. tholozani*, were identified. The collected ticks were individually preserved in 96% ethanol and stored at –20 °C until DNA extraction.

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DNA Extraction

Ticks designated for molecular analyses were washed three times in sterile water baths and subsequently crushed individually in clean 1.5-mL plastic tubes using sterile scalpels. Genomic DNA was then extracted and purified using the Genomic DNA Purification Kit (Cinaclon, Iran, Alborz) in accordance with the manufacturer's instructions. The quality and quantity of the extracted genomic DNA were assessed using a NanoDrop ND-1000 (NanoDrop Technologies) and agarose gel electrophoresis. DNA extracts were stored at –20 °C until further analysis.

GlpQ-PCR

In a previous study, we developed a PCR assay targeting a specific 219 bp fragment of the *glpQ* gene, which was successfully employed to detect Tick-borne Relapsing Fever Borreliae (20). In the present study, the same primer sequences (*F3-glpQ* and *B3-glpQ*) (Table 1) and amplification conditions were used to detect *Borrelia* DNA in the extracted samples. Briefly, the PCR reaction mixture (25 µl) comprised 3 mM $MgSO_4$ (Biobasic, Toronto, Canada), 1.6 mM dNTPs (Kawsar Biotech Co, Tehran, Iran), 1.5 µl of 10X buffer (containing 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl [pH 8.75], 1% Triton X-100, and 1 mg/ml BSA; Biobasic, Toronto, Canada), 1 U *Taq* DNA Polymerase (Biobasic, Toronto, Canada), 0.4 µM of each primer, 1 µl template DNA, and 8.5 µl double-distilled water (DDW). Amplification was performed in a thermal cycler (Eppendorf, Hamburg, Germany) programmed for an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 45 seconds, 45 °C for 45 seconds, and 72 °C for 30 seconds, with a final extension at 72 °C for 10 min. A no-template control, containing nuclease-free water in place of DNA, was included in each PCR run as a negative control, while positive control reactions containing the pTZ57R/T-*glpQ* recombinant plasmid were also employed. PCR products were resolved by electrophoresis on a 2% agarose gel (Min Run Gel Electrophoresis System; Bio-Equip Co., Shanghai, China), stained with ethidium bromide (CinnaGen, Alborz, Iran), and visualized under UV light using a Gel Documentation system (E-BOX VILBER, Marne-la-Vallée, France). A positive PCR product obtained from one tick DNA sample was purified using a PCR Purification Kit (Bioneer, Daejeon, Korea) and sequenced bidirectionally (ABI 3730xl/Bioneer 3730xl, Daejeon, Republic of Korea).

GlpQ-LAMP

In a previous study, we reported the initial design and implementation of a LAMP assay (*glpQ*-LAMP) for the rapid detection of tick-borne relapsing fever *Borrelia* (20). In the present study, we utilized the same reaction conditions and primer sets to detect *Borrelia* DNA in extracted genomic DNA from soft tick specimens. Each reaction mixture (total volume 25 µl) contained 40 pM of the inner primers (*glpQ*-FIP and *glpQ*-BIP; Table 1), 10 pM each of the outer primers (*glpQ*-F3 and *glpQ*-B3) and the loop primers (*glpQ*-LF and *glpQ*-LB), 11.2 mM dNTPs (Kawsar Biotech Co, Tehran, Iran), 0.8 M betaine (Sigma-Aldrich, Taufkirchen, Germany), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 0.05% Triton X-100 (Biolabs, New England, UK), 8 mM MgSO4

Table 1. The sequence of the primers to amplify a 219 bp fragment of *glpQ* gene of TBRF *Borrelia* using LAMP or PCR.

Primer	Sequence (5′–3′)	Reference
F3-*glpQ*	AATGCACGATCCTGAACT	(20)
B3-*glpQ*	TCTTCTTCTAGGGTTGGAATT	
FIP-*glpQ*	TGCTAATGTGAAATCGACGGAATAA-CAACAACAAATGTTGCAAAGC	
BIP-*glpQ*	AATCACTAAGCCTTAGCGAAAGAT-TGTTGCAGGAAAACGGTTA	
LF-*glpQ*	TCTCTAGCTCTTCCTGGAAACA	
LB-*glpQ*	CCTGAAACACAACAACCAATATACC	

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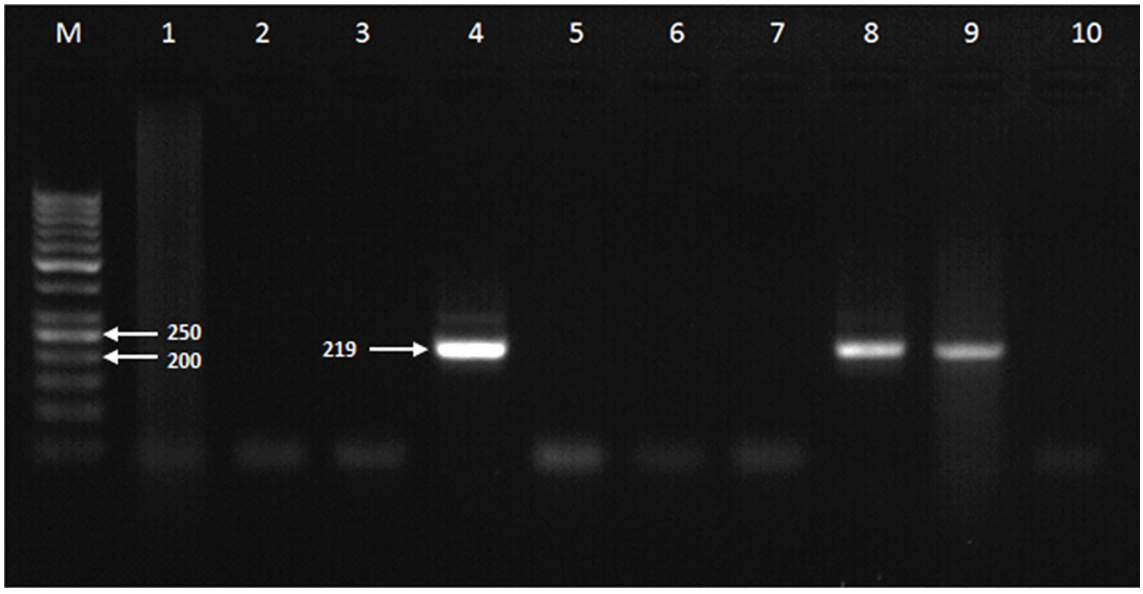


Figure 1. Agarose gel electrophoresis (2% w/v) of PCR products amplified by F3-*glpQ*, B3-*glpQ* primers. Lane M, 50 bp DNA ladder; lanes 1-8, tick DNA extracted from tick samples (a 219 bp band in lanes 4 and 8 indicates amplification of *glp-Q* fragment in *Borrelia* DNA); lane 9, positive control (pTZ57R/T-*glpQ* plasmid); lane 10.

(Biobasic, Toronto, Canada), 0.1% Tween 20 (Acros Organics, Vernon, USA), 8 U of Bst DNA polymerase (large fragment; Biolabs, New England, UK), 1 µl of template DNA, and 8 µl of deionized distilled water (DDW). The reactions were incubated at 65°C for 60 min using a Loopamp real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan), followed by heating at 80°C for 5 min to inactivate the enzyme (20). Positive and negative control reactions were also performed as described previously.

Detection of *glpQ*-LAMP Products

Amplification products were assessed using three methods: (1) direct visual observation for white turbidity resulting from the accumulation of magnesium pyrophosphate, a by-product of the reaction; (2) real-time measurement of optical density at 650 nm using a Loopamp real-time turbidimeter, with samples considered positive when turbidity reached ≥0.1 within 60 min; and (3) gel electrophoresis of the amplicons on 2% agarose gels.

Statistical Analysis

A Chi-square test and an agreement analysis were performed to compare the results of the *glpQ*-PCR and *glpQ*-LAMP assays.

Results

GlpQ-PCR and _glpQ_-LAMP

A total of 103 DNA extracts from collected ticks were analyzed. The *glpQ*-LAMP assay detected TBRF *Borrelia* DNA in 18.44% (19/103) of the samples, compared with 12.62% (13/103) detected by the *glpQ*-PCR assay (Figures 1 and 2). For both assays, the amplification results were consistent with those obtained from the positive control containing the pTZ57R/T-*glpQ* recombinant plasmid. BLASTn analysis of the sequenced PCR product against the NCBI databases confirmed that it corresponds to a fragment of the TBRF *glpQ* gene. The sequence has been deposited in the NCBI database as *glpQ Borrelia* sp. (GenBank accession no. MH188922). The Chi-square test indicated that the *glpQ*-LAMP assay was significantly more sensitive than the *glpQ*-PCR assay (P = 0.000). The agreement between the *glpQ*-LAMP and *glpQ*-PCR assays was nearly perfect (kappa=0.779). These results suggest that the *glpQ*-LAMP assay is a reliable tool for detecting TBRF *Borrelia* DNA in infected ticks.

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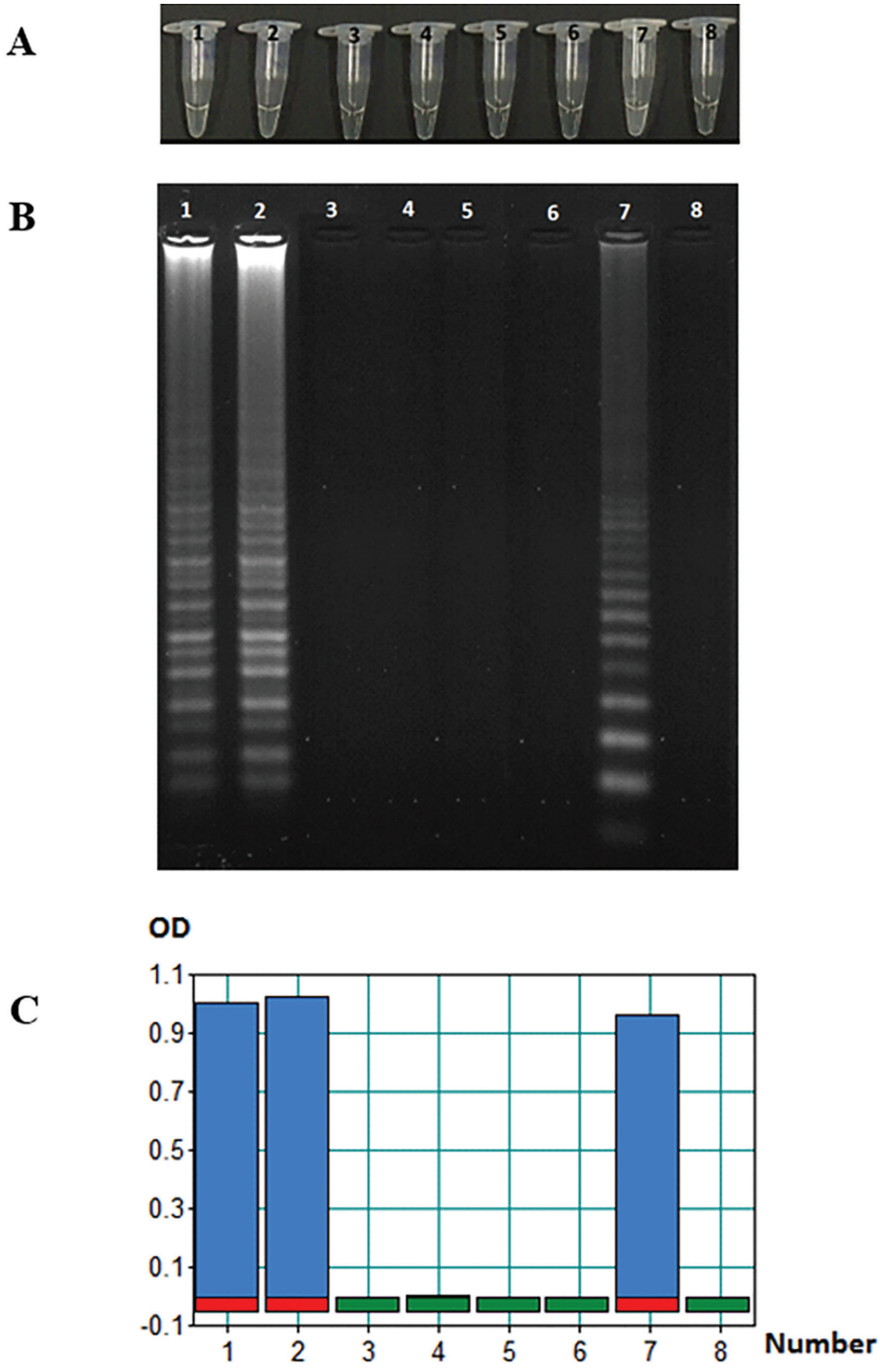


Figure 2. Loop-mediated isothermal amplification (LAMP) experiments were conducted on tick DNA samples. Panel A illustrates the visual appearance of white turbidity in the LAMP reactions: tubes 1 and 2 represent positive samples; tubes 3–6 denote negative samples; tube 7 serves as the positive control (pTZ57R/T-*glpQ* plasmid); and tube 8 functions as the negative control (DDW). Panel B displays the agarose gel electrophoresis of the LAMP products, with lanes 1 and 2 corresponding to positive samples, lanes 3–6 to negative samples, lane 7 to the positive control (pTZ57R/T-*glpQ* plasmid), and lane 8 to the negative control (DDW). Panel C presents the amplification bar chart generated by the Loopamp real-time turbidimeter, with the sample order identical to that in Panels A and B.

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Discussion

There exists a specific relationship among *Borrelia* species in each endemic region (21). The prevalence and transmission of TBRF constitute significant public health concerns. Efforts to control these emerging diseases have been hampered by challenges in managing tick populations as well as in detecting and treating the infections caused by the transmitted pathogens (22). Given the widespread distribution of tick vectors in endemic areas worldwide, TBRF likely represents an important animal health issue that should be investigated and considered as a differential diagnosis in the management of febrile patients exhibiting similar clinical symptoms (23). The choice of molecular method can substantially influence the detected prevalence of the pathogen (24). For instance, because the spirochetemia phase is brief and laboratory detection is heavily dependent on the observer's expertise, TBRF is frequently underdiagnosed even in regions where clinical suspicion should be high. Incorporating routine molecular testing to detect spirochetes could help eliminate these diagnostic uncertainties in rural settings (21).

In Iran, the incidence of TBRF appears to vary regionally. Although the national prevalence is reported at slightly over 200 cases per year (25), this figure likely underestimates the true prevalence, as many cases remain misdiagnosed or unrecognized (7). In tropical and subtropical regions, both malaria and TBRF occur, and because fever is the predominant symptom in both diseases, TBRF is often overlooked or misdiagnosed as malaria, resulting in inappropriate treatment (26).

Various techniques have been employed for the detection of *Borrelia* species to date. Owing to the spirochetes' slender and transparent morphology, detection via conventional light microscopy is exceedingly challenging and of limited utility. The low sensitivity of microscopic tests necessitates the development of more accurate diagnostic methods. Moreover, culture and isolation of *Borrelia* require approximately 21 days, which is impractically lengthy, while serological tests are hampered by a high rate of false-positive results and an inability to differentiate between active and past infections (26).

In Iran, PCR assays based on various molecular markers—such as *glpQ*, *rrs*, and *flaB*—have been developed for the detection of *Borrelia* infection in ticks (27, 28) or for characterizing tick-borne relapsing fever *Borrelia* (9, 26); however, clinical samples have seldom been included in these studies (20). In our previous study, we developed a LAMP assay targeting the *glpQ* gene (20). Blood samples from patients suspected of relapsing fever and DNA extracted from dried blood spots (DBSs) of spirochetemic mice were evaluated using this novel method, and the results were comparable to those obtained by the standard Borrelia-specific nested PCR. Our subsequent objective was to assess the utility of the *glpQ*-LAMP assay in detecting *Borrelia* in suspected tick samples. In the current study, 103 field-collected ticks from the Qorveh district of Kurdestan Province were analyzed to evaluate the performance of the *glpQ*-LAMP assay under isothermal conditions for the amplification of relapsing fever *Borrelia* (RFB) DNA, in comparison with *glpQ*-PCR. The results revealed that an average of 18.44% of ticks tested positive for RFB, thereby confirming the presence of the pathogen in this region as determined by the *glpQ*-LAMP assay. Moreover, the *glpQ*-LAMP results were consistent with 13 positive and 84 negative samples as identified by *glpQ*-PCR. Notably, six samples that tested positive by *glpQ*-PCR were negative according to the *glpQ*-LAMP assay. Nevertheless, the marked difference in positive detection rates between the two methods is likely attributable to the use of six specific primers, which confer enhanced sensitivity to the LAMP assay.

Advantages of LAMP include its simplicity

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and capacity to generate large volumes of amplified products, which can be readily visualized by the turbidity of the reaction mixture. In Iran, 1,415 cases of TBRF have been reported between 1997 and 2006, with the highest incidence observed in Ardabil (44.2%), followed by Hamedan (15.4%), Zanjan (12.9%), and Kurdestan (9.8%) provinces (11). Our findings revealed a higher rate of positive detections using the LAMP method compared to PCR, corroborating our previous results obtained with glpQ-LAMP in detecting Borrelia in blood samples from patients suspected of relapsing fever (20).

In China, the LAMP assay demonstrated superior sensitivity over conventional PCR in detecting *Borrelia burgdorferi* sensu lato in ticks (29). Moreover, a LAMP assay developed for the spotted fever group of *Rickettsia* exhibited a sensitivity that was tenfold greater than that of end-point PCR (30). Consequently, LAMP may serve as an effective tool for monitoring TBRF or for screening ticks in regions where the disease is endemic (31). These findings suggest that an increasing number of tick species are involved in maintaining *Borrelia* pathogens in nature (29). A study conducted in West Africa in 2005 reported an incidence as high as 11% in the population, indicating that TBRF is a common cause of fever in many rural areas of the region (32).

Conclusion

The *glpQ*-LAMP assay is more sensitive than glpQ-PCR for detecting TBRF *Borrelia*. Unlike PCR, LAMP offers the dual advantages of simplicity and cost-effectiveness. Accordingly, the *glpQ*-LAMP method represents a valuable alternative molecular diagnostic tool for RFB infections in field studies conducted in low-resource settings. Moreover, field-collected tick samples, specimens from rural health centers, and suspected blood samples can be efficiently screened for RFB using minimal equipment, such as a heat block maintained at 65°C.

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Conflict of Interest

All authors declare that they have no conflicts of interest.

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Ethical Considerations

The study received approval from the Human Research Ethics Committee of AJA University of Medical Sciences.

Code of Ethics

IR.AJAUMS.REC.1399.009

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