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Detection of *Rickettsia* in ticks using Loop-mediated Isothermal Amplification (LAMP)

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Abstract

Objectives

The objective of the study was to develop a Loop-mediated Isothermal Amplification (LAMP) assay for screening of *Rickettsia* species circulating in ticks using the citrate synthase gene (*gltA*). The LAMP assay employed portable visualisation methods, making the assay more field-suitable. Furthermore, prior methods have not used *gltA* as the target, despite proven success in Polymerase Chain Reaction (PCR) methods.

Methods

Using an alignment of 72 DNA sequences (comprised of 21 *Rickettsia* species) from GenBank we designed a novel set of *gltA* LAMP primers. Evaluation used DNA from 12 *Rickettsia* species as positive controls (extracted from cultures or naturally infected ticks) alongside a panel of negative controls representing different bacterial species. Subsequently this assay was used to screen 295 *Ixodes ricinus* and 24 *I. hexagonus* ticks collected from the UK (including northern and southern England and northern Scotland).

Results

LAMP successfully detected 11 out of 12 (91.7%) *Rickettsia* species, excluding *Rickettsia akari*. Among 319 ticks collected in the UK, three were positive for *Rickettsia* (0.9%). All three positives were *I. ricinus* ticks, while none of the 24 *I. hexagonus* ticks were positive. Results were confirmed using a published PCR

method. Sanger sequencing of PCR amplicons generated for each positive tick showed that they were all *R. helvetica.*

Conclusions

This study introduces a novel field-applicable LAMP protocol for efficient *Rickettsia* screening in ticks to better assess its prevalence and consequent health risks. Furthermore, this assay has proven suitability for rickettsial detection in *I. ricinus* ticks, which has been reported as unsuccessful in previous European studies.

Key words: *Rickettsia*; LAMP; tick; *Ixodes ricinus*; field

1. Introduction

Rickettsia are Gram-negative intracellular bacteria that can cause disease in humans. Transmission typically occurs through the bite of infected arthropods, particularly ticks, fleas, and lice. Human cases have been reported across many European countries, including Spain, Portugal, Italy, France, and Eastern European nations [1-6]. Clinical presentations can vary by *Rickettsia* species, some causing mild illness, whilst others, such as *Rickettsia conorii* (Mediterranean spotted fever) can lead to severe or even fatal outcomes if untreated. The most common clinical triad comprises fever, rash and an inoculation eschar at the tick bite site [1-5]. However, in some clinical cases these signs might be absent leading to diagnostic challenges. Diagnostic approaches include serological testing (e.g., Indirect Immunofluorescence Assay, IFA), tissue culture isolation and molecular methods such as Polymerase Chain Reaction (PCR) which is particularly valuable for early

detection, as antibody-based methods are less reliable during acute infection [6]. In Europe, ticks are becoming increasingly recognised as both vectors and reservoirs for *Rickettsia*, contributing to the epidemiology of emerging and re-emerging rickettsial infections. The need for active surveillance of rickettsial infections, alongside other tick-borne pathogens, is crucial for monitoring infection patterns, assessing disease burden, and evaluating risk factors for human and animal health [7].

To date, molecular rickettsial screening has relied upon PCR targeting multiple genes, including citrate synthase (*gltA*), and outer membrane proteins A and B (*OmpA* and *OmpB*) [8,9]. These genes allow both detection and species-level differentiation. Expanding surveillance efforts and improving diagnostic methodologies are essential for early detection, timely intervention, and effective public health responses.

PCR-based diagnostics are labour-intensive, time-consuming and require specialised equipment in a lab setting [10] making them largely unsuitable for use in the tick's natural environment. DNA amplification using Loop Mediated Isothermal Amplification (LAMP) can overcome some of the limitations of PCR, whilst preserving both diagnostic sensitivity and specificity. LAMP utilises an isothermal reaction (eliminating the need for a thermocycler) together with six primers, culminating in enhanced sensitivity, specificity and faster reaction speed. Amplification results can be visualised either as colorimetric change, real-time fluorescence peaks or turbidity change [10,11], making this a more flexible approach which can be adapted to suit either the field setting or resource poor environments.

Surprisingly, the *gltA* gene has not been used as a LAMP target to date, despite its proven track record in PCR assays [8,12]. Previous LAMP detection designs have used limited rickettsial sequence alignments for primer design (11 *Rickettsia* species or less) potentially compromising the detection of the full rickettsial diversity. Furthermore, previous LAMP assays have largely been applied to American tick populations, with limited relevance to Europe. The only LAMP assay used to screen European ticks from Italy [13] failed to detect any *Rickettsia* in *I. ricinus* ticks (despite a positive in parallel PCR testing), suggesting its incompatibility with European rickettsial species. Finally, field suitability of previous LAMP assays has been limited, with none using portable amplification detection methods.

To overcome these limitations, we developed and tested a novel, field-suitable LAMP assay for *Rickettsia* detection in *I. ricinus* and *I. hexagonus* ticks from the UK, using the *gltA* gene target. This assay has potential for Spotted Fever Group (SFG) *Rickettsia* screening of *I. ricinus* and *I. hexagonus* ticks as well as for other tick species and in other European locations.

2. Methods

2.1 Primer design

GenBank was searched for rickettsial *gltA* sequences. Full search terms and inclusion/exclusion criteria can be found in the supplementary files (S1). For *Rickettsia* species with more than five entries, only the first five were exported (to reduce bias towards more frequently-reported species).

In total, 114 sequences, covering 33 *Rickettsia* species across SFG, Typhus group (TG), Ancestral (AGR) and Transitional (TGR) groups (listed in Table 1), were exported and aligned using ClustalW within MEGA 11 software. The alignment was manually inspected to identify areas of poor conservation, and species which contributed significantly to this were removed. This process resulted in removal of 12 *Rickettsia* species, leaving 21 SFG *Rickettsia* species (summarised in Table 1). The conserved sequence alignment was exported into LAMP Designer version 1.16 (Premier Biosoft) and used to generate multiple primer sets from which the best option was selected for further use (provided in Table 2).

2.2 Preparation and testing of positive and negative control DNA

Primers were initially tested using positive control DNA extracted from *Rickettsia* cultures and *Rickettsia*-positive ticks (described in Table 3). Assay sensitivity was assessed by calculating the lower detection limit of the primers (described in S2). To assess specificity, DNA from control bacteria found commensally in ticks [14] was tested (see S3).

LAMP primer mixes were prepared according to a New England Biolabs protocol [15]. Briefly, three primer mixes were prepared, with primer mix 1 containing 5 µM F3 and 5 µM B3, primer mix 2 containing 40 µM FIP and 40 µM BIP and primer mix 3 containing 10 µM LF and 10 µM LB.

LAMP reactions using control samples were performed according to Optigene guidelines [16]. Briefly, a 25 µL reaction mix was prepared containing 1 µL of each

primer mix, 6 µL nuclease-free water, 15 µL GspSSD2.0 Isothermal Mastermix ISO-004 (Optigene) and 1 µL of DNA template. Each reaction was incubated at 65°C for 30 minutes using Genie II LAMP machine (Optigene) and outputs were read as fluorescence peaks. All positive and negative control DNA extracts were tested in duplicate.

2.3 UK tick population screening

A total of 319 ticks were obtained from across the UK between 2022-2023. Blanket dragging was used for collection of 214 of these, and the rest were from veterinary sources. A summary of collection location, time, vertebrate host species (where relevant), tick species and life stage is presented in the supplementary files (Table S4). Morphological identification was undertaken for tick species confirmation according to a previously published guide [17]. Particular attention was paid to the scapular plate and internal spur (from coxae I) to differentiate species. A total of 295 were identified as *I. ricinus*, whilst the remaining 24 were identified as *I. hexagonus*.

DNA was extracted from individual ticks using Instagene Matrix (Biorad) according to manufacturer instructions, except for the incubation length (extended to one hour for both temperature steps as whole tick specimens were used). Molecular corroboration of tick identity was undertaken with one in 10 samples being subjected to conventional PCR. Further details can be found in the supplementary files (S5).

For rickettsial screening, aliquots of individual DNA extracts were combined into a 20 µL pool containing 10 individual samples (ticks 1-10, 11-20, etc) using 2 µL from each sample. A total of 32 DNA pools were prepared (31 pools of 10 ticks, and one

pool of nine ticks). Each pool was tested using the fluorescence LAMP assay (described above) and quantitative real-time PCR (qPCR) in duplicate. PCR recombinant Taq DNA Polymerase kit (Invitrogen) reagents were used as previously described [18]. The *gltA* primer and probe were used as previously described [19]. The qPCR amplifications used an AriaMX Real-time PCR System (Agilent) with cycling conditions as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Pooled samples detected using both qPCR and LAMP in duplicate were reported as positive. For these, each sample within the positive pool was tested individually using qPCR and LAMP (in duplicate) as above to identify individual positives. Individual tick samples positive on both qPCR and LAMP duplicate runs were considered positive.

Rickettsia species identity was determined using conventional PCR in a final volume of 25 µL. Reaction conditions, sequencing and identification were as described for tick identification but using LAMP primers F3 and B3 at a final concentration of 500 nm (see Table 2). A 300 bp amplicon was generated for each positive sample and Sanger sequencing performed as described in the supplementary files (S5).

3. Results

3.1 Assay development

Our LAMP primers detected seven *Rickettsia* species out of eight serving as positive control DNA (Figure 1), only failing to detect *R. akari*. The LAMP primers also successfully detected *R. rickettsii* (see S6). The lower detection limit (sensitivity) of the assay was 0.025 ng/µL (see S7). Subsequently the assay was evaluated for its

ability to detect *Rickettsia* in known-positive European ticks (*I. ricinus*, *Dermacentor marginatus* and *Rhipicephalus sanguineus*) covering six *Rickettsia* species. The LAMP primers amplified *Rickettsia* from all tick samples evaluated (Figure 2). Finally, a range of non-target pathogen species were tested to ensure that these were not detected by the LAMP assay, with none yielding reactivity (shown in Figure S6).

3.2 Screening of UK ticks

Of the 32 DNA pools tested (containing 319 ticks obtained from the UK, as summarised in Table S4), three pools showed a positive result on both qPCR and LAMP. Individual ticks from these three positive pools were then tested to identify which specific tick was positive. Three individual ticks were positive on both qPCR and LAMP, and all three of these ticks were *I. ricinus*. Two of these were unfed adult field-collected ticks in Surrey and the final tick was a partially fed adult collected from a dog in Sussex. The 300 bp amplicon generated from each positive tick was sequenced and all identified as *R. helvetica*. None of the 24 *I. hexagonus* ticks were positive for *Rickettsia* by either qPCR or LAMP.

4. Discussion

Our genus-specific LAMP assay successfully detected the presence of *R. helvetica* within *I. ricinus* ticks screened in the UK (3/319; 0.9%). *R. helvetica* is classified as a human pathogen, albeit mild in most cases [20] with cases of aneruptive fever, subacute meningitis and cardiac involvement described [21].

Previous UK studies for *Rickettsia* have screened ticks by PCR. A study carried out in Northwest Scotland collected *I. ricinus* ticks from 26 sites (n=2828) which were tested using a *gltA* PCR for *R. helvetica*, but found just one of the ticks positive 1/2828 (0.04%) [12]. A greater geographical range was evaluated in a study of 338 ticks collected from various UK locations [22]. Using a generic *Rickettsia*-specific *16S rRNA* PCR [23] 22 of 338 *I. ricinus* ticks were positive for *Rickettsia* – including ten ticks infected with *R. helvetica* (3%). Their findings are largely consistent with ours, although we did not detect any of the other species found in this previous work (*R. limoniae*, *R. massiliae*, *R. canadensis* and *R. bellii*).

Prior LAMP studies for *Rickettsia* detection in ticks, have shown mixed success. For example, one study aligned *17-kDa* gene sequences from four *Rickettsia* species (*R. rickettsii*, *R. amblyommatis*, *R. parkeri* and *R. montanensis*). LAMP primers were designed and evaluated using DNA extracted from seven SFG and one TG species. These primers detected all positive control species (except for *R. typhi*) [24]. This LAMP assay was subsequently used (in parallel with end-point PCR) to screen 186 field-collected ticks from Oklahoma City (*Amblyomma americanum*, *D. variabilis* and *A. maculatum*) allocated into 40 pools. Results demonstrated both PCR and LAMP successfully detected *R. amblyommatis*, *R. montanensis*, *Candidatus R. andeanae* and *R. felis*.

Hanaoka et al [25] aligned 12 complete genome sequences across 10 *Rickettsia* species, using this for LAMP primer design. Using turbidimetry measurements, only one LAMP primer set out of five successfully detected all 12 test species (10 SFG and two TG). Others designed LAMP primers using the *OmpB* gene following

alignment of 11 *Rickettsia* species [26] with amplification assessed using turbidity measurements and gel electrophoresis. The assay detected all SFG *Rickettsia* species evaluated but failed to detect TG members. In parallel with PCR, Raele et al [13] used these *OmpB* LAMP primers reported above [26] to screen 158 ticks collected in Italy (including *I. ricinus* among other species). PCR detected *R. helvetica* in one of the *I. ricinus* pools but the LAMP performed poorly, failing to detect *R. helvetica* in these *I. ricinus* ticks, and yielded inferior detection with other rickettsiae in different tick species.

We report the first time that a LAMP assay has been successfully used to detect *Rickettsia* in *I. ricinus* ticks in Europe. Our investigations also showed the capacity of our LAMP assay to detect other SFG rickettsial species both with control cultures and known infected ticks, including other *Rickettsia* species commonly reported in Europe (such as *R. monacensis, R. raoultii* and *R. slovaca*) [4].

A key strength of our work was that design of the LAMP primers utilised a larger set of aligned sequences improving robustness compared to previous studies. Furthermore, our LAMP assay is a more field-suitable approach than previous studies, using a portable LAMP amplification method (Genie II) which can be applied in the field setting with no power source, alongside Instagene Matrix (Biorad) which requires minimal resources for DNA extraction, offering excellent potential for use of this assay in resource-poor settings. Our protocol provides a *Rickettsia* screening result in less than three hours after collection of the tick, offering further advantages. A natural extension of this assay would be to evaluate its performance with samples

taken from clinical eschar lesions where it can provide a considerable improvement in timely and appropriate patient management.

However, the challenge for any genus-specific rickettsial assay targeting rickettsial detection in ticks, is the considerable presence of rickettsial endosymbionts in ticks [27]. To fully exclude detection of these in a reactive sample would necessitate further molecular analysis to determine the rickettsial species present. The genus *Rickettsia* contains a wide array of species, many only characterised by molecular approaches and consequently only have *Candidatus* taxonomic status [28]. A further limitation of our study was the small sample size. We have conducted a pilot study with 319 ticks of limited species, all collected from the UK in a restricted geographical region and consequently may not represent the diversity within the Rickettsiae. Furthermore, heterogeneity has been noted even within *gltA* of *R. helvetica* from a single country [21]. Future work providing geographical expansion and inclusion of other tick species coupled with increased sample size would enhance performance evaluation.

Our pilot data has confirmed success of our LAMP assay in a sample of ticks from the UK, however, more comprehensive parallel testing alongside qPCR would improve the validation of this assay as a screening method for detection of Rickettsiae in a wider range of locations in Europe and beyond. An increasing clinical impact of rickettsiosis has been noted in some locations, providing further justification for surveillance. For example, a recent study carried out in Spain examined clinical cases of *R. sibirica mongolitimonae* at a diagnostic centre between 2007-2024, with results showing an increased number of cases over time [29].

Furthermore, a recent Canadian study of hospitalisations following Lyme disease and other tick-borne diseases between 2009 until 2021, noted that of those with other tick-borne infections (162), that 32.7% of these were rickettsiosis and noted that the rickettsial annual incidence had increased considerably over the study period [30].

In conclusion, this study reports on a field-suitable *Rickettsia* detection assay, which will aid tick surveillance and provide key insights into *Rickettsia* prevalence to ultimately guide intervention approaches to public health in the UK and wider Europe.

Transparency declaration

Conflict of interest

Roberto La Ragione is a Scientific Advisor to Vidiia. There are no further conflicts of interest to declare.

Acknowledgements

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Author contributions

Project conception and funding was achieved by SC, RL, MB and MN. Delivery of the laboratory work was primarily carried out by SL, with all team members contributing towards optimisation, troubleshooting and/or laboratory work. MH provided extensive guidance and training in all aspects of the LAMP assay (including primer design, assay performance optimisation and intellectual troubleshooting advice). *Rickettsia* control samples and specialised advice were provided by RS. The manuscript was drafted by SL and SC and edited by all team members. All authors approved manuscript submission.

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Ethical considerations

Based on completion of a University of Surrey SAGE-AR form (ID 638929-638920-93244722), no formal ethical approval was required for UK veterinary surgeons to provide ticks collected from animals as part of routine veterinary practice for the project. Tick collection permits were obtained for each blanket dragging area.

Main body figure legends

Graphical abstract
Created in BioRender. Cutler, S. (2025) https://BioRender.com/m74g606

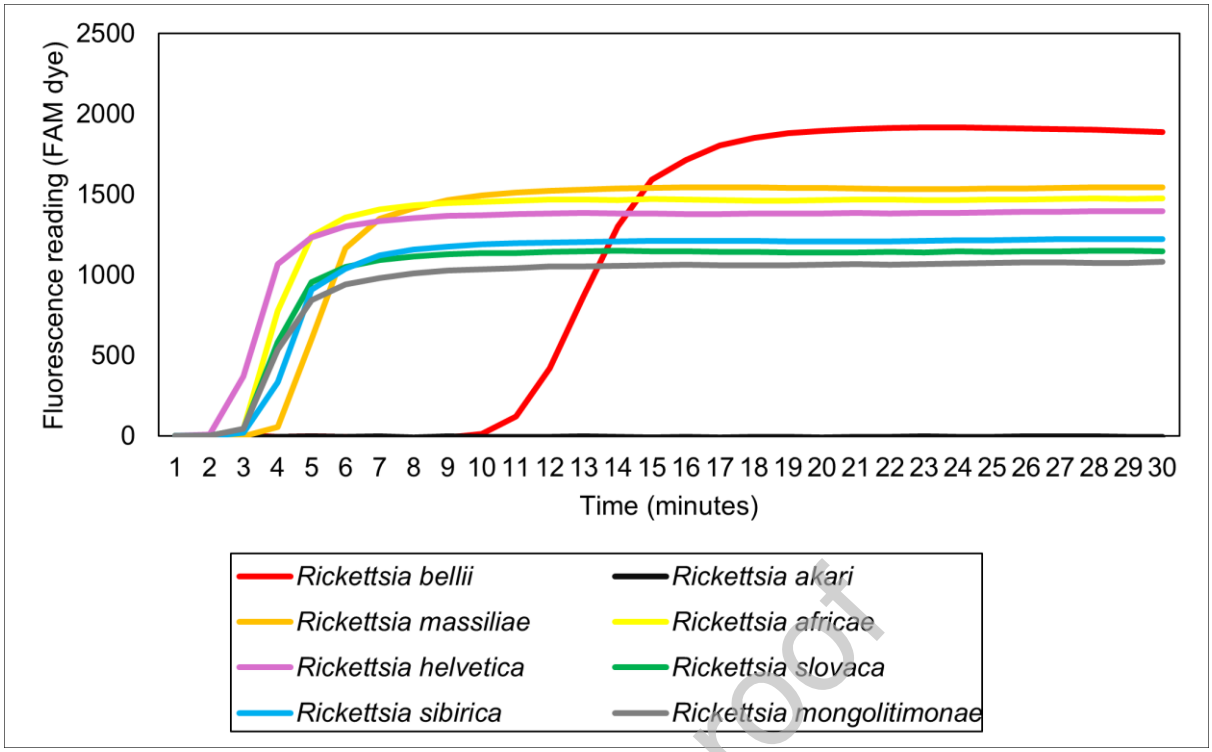

Figure 1: Fluorescence LAMP primer testing results for *Rickettsia* DNA only
The results show that the LAMP primers can detect Spotted Fever group *Rickettsia* (*R. massiliae, R. africae, R. helvetica, R. slovaca, R. sibirica* and *R. sibirica mongolitimonae strain*) and Ancestral group *R. bellii* (but with later amplification for this species). The primers were unable to detect *R. akari* (from the Transitional group).

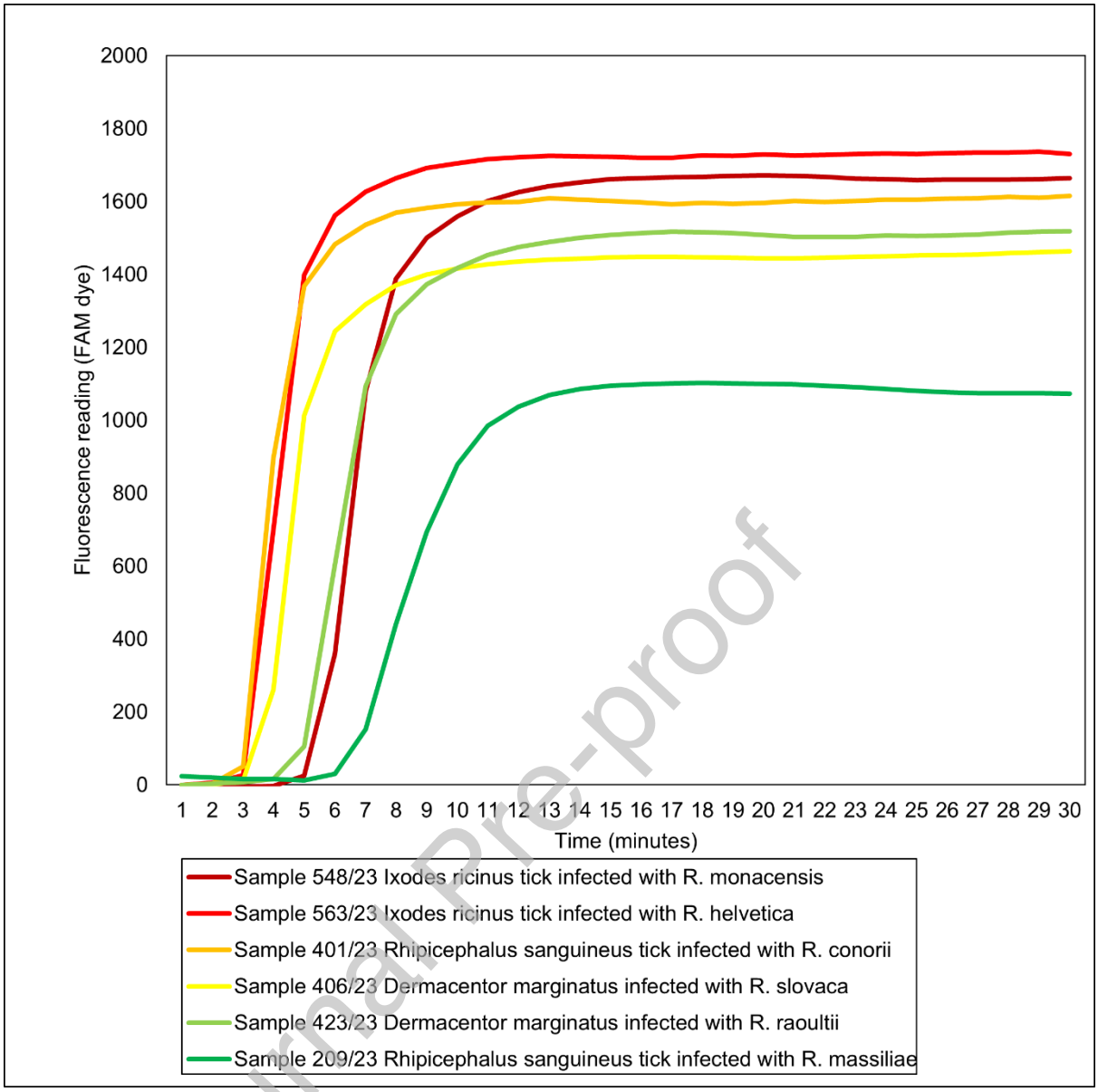

Figure 2: Fluorescence LAMP primer testing results for known *Rickettsia*-positive *Ixodes ricinus*, *Rhipicephalus sanguineus* and *Dermacentor marginatus* tick samples

The LAMP primers were able to detect *Rickettsia* DNA present in three different tick species (*Ixodes ricinus*, *Rhipicephalus sanguineus* and *Dermacentor marginatus*) and across all of the *Rickettsia* species tested (*R. monacensis*, *R. helvetica*, *R. conorii*, *R. slovaca*, *R raoultii* and *R. massiliae*).

Main body table titles

***Rickettsia* species used in alignment (21)**	***Rickettsia* species removed from alignment (12)**

Species	Group	Species	Group
Rickettsia africae	Spotted Fever	*Rickettsia akari*	Transitional
Rickettsia aeschlimannii		*Rickettsia asembonensis*	Transitional
Rickettsia amblyommatis		*Rickettsia australis*	Transitional
Rickettsia asiatica		*Rickettsia hoogstraalii*	Transitional
		Rickettsia bellii	Ancestral
Rickettsia conorii		*Rickettsia canadensis*	Ancestral
Rickettsia heilongjiangensis		*Rickettsia monteiroi*	Ancestral
		Rickettsia gravesii	Spotted Fever
Rickettsia honei		*Rickettsia helvetica*	Spotted Fever
Rickettsia japonica		*Rickettsia tamurae*	Spotted Fever
Rickettsia marmionii			
Rickettsia massiliae		*Rickettsia prowazekii*	Typhus
Rickettsia monacensis		*Rickettsia typhi*	Typhus
Rickettsia mongolitimonae			
Rickettsia montana			
Rickettsia parkeri			

Rickettsia peacockii		
Rickettsia raoultii		
Rickettsia rhipicephali		
Rickettsia rickettsii		
Rickettsia sibirica		
Rickettsia slovaca		
Rickettsia vini		

Table 1: Summary of *Rickettsia* species aligned using Mega11 for LAMP primer design

Primer	Nucleotide sequence
Forward 3 (F3)	TGCAATGCCTTGTACGAA
Backward 3 (B3)	GCCTATTAACCTAAACGGATCA
Forward Inner Primer (FIP)	GGTTAGCTCCGGATGAGCCGCCGATCATGAGCAGAAT
Backward Inner Primer (BIP)	CTTTGGGGACCTGCTCACGGGATATTCTCAGAACTACCGAT
Loop Forward (LF)	TCGGACTGTTGAAGTAGAAGC
Loop Backward	GGGGCTAATGAAGCGGTAA

(LB)	

Table 2: LAMP primer set designed using LAMP Designer software

***Rickettsia* DNA species**	**DNA extracted from:**	**Source information**
R. rickettsii	Culture	Culture passage of isolates provided to Dr. Fátima Bacellar from the University of Texas Medical Branch, Galveston (UTMB), TX, United States
R. bellii	Culture	
R. akari	Culture	
R. africae	Culture	
R. massiliae	Culture (isolated from tick specimen)	Provided by Center for vectors and infectious diseases, Águas de Moura
R. mongolitimonae	Culture (isolated from human blood)	
R. helvetica	Culture (isolated from tick specimen)	
R. slovaca	Culture (isolated from human blood)	
R. sibirica	Culture	Provided by Unité des Rickettsies, CNRS UMR 6020. Faculté de Médecine de Marseille, France.
R. conorii	Tick 401/23 (*R. sanguineus*)	Ticks obtained from the Portuguese National Surveillance Network
R. slovaca	Tick 406/23 (*D. marginatus*)	
R. raoultii	Tick 423/23 (*D. marginatus*)	
R. monacensis	Tick 548/23 (*I. ricinus*)	
R. helvetica	Tick 563/23 (*I. ricinus*)	
R. massiliae	Tick 209/23 (*R. sanguineus*)	

All positive control samples were confirmed using PCR (using the *gltA* target).

Table 3: Summary of positive control material used for LAMP primer testing

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Supplementary figure legends

S1: Table listing inclusion and exclusion criteria used in the *Rickettsia* sequence search on GenBank

S2: Details of the detection limits assay used to assess primer sensitivity

S3: Table listing the negative control bacterial species used for LAMP primer testing and associated information

S4: Table outlining the source information and associated details for each of the 319 ticks obtained from the UK

S5: Details of the conventional PCR protocol and sequencing process used for tick identification

S6: Graph showing the fluorescence LAMP primer testing results for negative control pathogens
The results show that the primers detect *R. rickettsii* (positive control) but do not detect the range of negative control pathogens tested.

S7: Graph showing the fluorescence LAMP detection limits (sensitivity) of the LAMP primers

The results show that when using the fluorescence LAMP primers to test for *R. rickettsii* at varying concentrations between 1 ng/µL and 0.0125 ng/µL, the primers are able to detect *Rickettsia* DNA at a concentration of 0.025 ng/µL and above. The 0.0125 ng/µL concentration was not detected by the primers.

Declaration of interests

☐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.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract