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Development and Clinical Validation of a Rapid RPA Assay for Ehrlichia Canis Detection in Canine Blood Using Fluorescence and Lateral Flow Formats

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Abstract

Ehrlichia canis, the causative agent of canine monocytic ehrlichiosis (CME), is a globally important tick-borne pathogen in dogs. Current diagnostic methods such as PCR require advanced laboratory infrastructure, limiting their use in field settings. This study developed and validated a dual-format recombinase polymerase amplification (RPA) assay targeting the 16S rRNA gene of E. canis, incorporating both fluorescence-based real-time detection and lateral flow strip readouts. Primers and probes were designed using Primer-BLAST and optimized for RPA at 39 °C for 20 minutes. Analytical sensitivity reached 10 femtograms of genomic DNA (~7–10 bacterial genomes), with 100% analytical specificity against related pathogens (Anaplasma platys, A. phagocytophilum, Rickettsia rickettsii). Clinical validation with 500 canine blood samples from Chennai, India, identified 43 positive cases (8.6% prevalence), achieving perfect concordance with conventional PCR (100% sensitivity and specificity; Cohen's κ = 1.000). The fluorescence format provided semi-quantitative results with strong correlation between DNA concentration and threshold time (R^2 = 0.981), while the lateral flow format enabled simple visual interpretation within 30 minutes. The RPA assay demonstrated excellent reproducibility (CV < 7%), reagent stability at ambient temperature (>6 months), and ~65% cost reduction compared to PCR. These results highlight the RPA assay as a rapid, accurate, and field-deployable diagnostic tool for CME, particularly suited to veterinary practice and surveillance in resource-limited, tick-endemic regions.

Keywords: *Ehrlichia canis, recombinase polymerase amplification, canine monocytic ehrlichiosis, point-of-care diagnostics, lateral flow, fluorescence detection*

1. INTRODUCTION

Canine monocytic ehrlichiosis (CME) is a widespread and economically important tick-borne disease of dogs caused by Ehrlichia canis, an obligate intracellular rickettsial bacterium transmitted primarily by the brown dog tick, Rhipicephalus sanguineus. The disease is endemic in many tropical and subtropical regions, including India, where climatic conditions favor high tick burdens and facilitate the spread of infection [1]. CME presents with a wide spectrum of clinical manifestations ranging from acute fever, anorexia, and lymphadenopathy to chronic, life-threatening conditions characterized by severe anemia, thrombocytopenia, pancytopenia, and hemorrhagic syndromes. If not diagnosed and treated early, E. canis infections can result in high morbidity and mortality in companion animals, making timely diagnosis essential for effective clinical management and control. Conventional diagnostic approaches for CME include microscopic examination of Giemsa-stained blood smears, serological methods such as ELISA and indirect immunofluorescence antibody tests (IFAT), and molecular assays [2]. While blood smear examination is rapid and inexpensive, its sensitivity is low, particularly during the subclinical and chronic stages of infection. Serological methods improve sensitivity but often suffer from cross-reactivity with other closely related Ehrlichia and Anaplasma species, leading to false positives. Moreover, antibody-

based tests cannot reliably distinguish between past exposure and current infection, thereby limiting their diagnostic utility [3].

Molecular techniques, particularly polymerase chain reaction (PCR), have become the gold standard for the detection of E. canis due to their high sensitivity, specificity, and ability to detect pathogen DNA during all stages of infection. However, the widespread application of PCR in routine veterinary practice is hindered by several constraints. PCR requires sophisticated equipment such as thermal cyclers, well-equipped laboratories, uninterrupted electricity supply, and trained personnel [4]. These requirements make PCR unsuitable for rapid, point-of-care testing in field settings or small veterinary clinics, especially in resource-limited environments where CME is most prevalent. To overcome these limitations, isothermal amplification methods have been explored as alternatives to PCR. Loop-mediated isothermal amplification (LAMP) has gained popularity because of its sensitivity and robustness. Nonetheless, LAMP assays require complex primer sets (four to six primers), and the amplification process typically takes close to an hour to complete. Additionally, the assay's reliance on specialized reaction chemistry and risk of non-specific amplification restrict its broader utility outside research or specialized diagnostic centers [5]. Recombinase polymerase amplification (RPA) has recently emerged as a promising isothermal nucleic acid amplification technique that addresses many of these drawbacks. RPA operates at a constant low temperature (37–42 °C), eliminating the need for thermal cycling equipment. The reaction is rapid, typically achieving detectable amplification within 20–30 minutes. RPA is also highly tolerant of crude DNA extracts, reducing the dependency on elaborate sample preparation procedures. Detection can be achieved either by fluorescence monitoring in real-time platforms or by simple visual inspection using lateral flow devices, making the technology versatile and adaptable to both laboratory and point-of-care applications. Furthermore, RPA reagents are stable at ambient temperature, offering advantages in field deployment where cold-chain storage is often unavailable [6].

Considering the urgent need for rapid, accurate, and field-deployable diagnostics for CME, this study was designed to develop and validate an RPA assay targeting the 16S rRNA gene of E. canis. The assay was optimized in both fluorescence-based and lateral flow formats, evaluated for analytical sensitivity and specificity, and validated with canine blood samples collected from the Chennai region of India. The diagnostic performance of the RPA assay was compared with conventional PCR to assess its potential as a reliable and cost-effective alternative for the detection of E. canis in field and clinical settings.

2. MATERIALS AND METHODOLOGY

2.1 Sample collection

A total of 500 canine blood samples were collected from dogs presented to veterinary hospitals and private clinics in Chennai, Tamil Nadu, India, between [insert study period]. Dogs with a history of fever, anorexia, lethargy, or suspected tick infestation were included in the study. Approximately 2 mL of whole blood was drawn aseptically from the cephalic or jugular vein of each animal into EDTA-coated vacutainer tubes. All samples were transported on ice to the laboratory and stored at –20 °C until DNA extraction.

2.2 DNA extraction

Genomic DNA was extracted from 200 µL of whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA was eluted in 50 µL of elution buffer and stored at –20 °C until further analysis. DNA quality and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) [7].

2.3 Primer and probe design for RPA

Primers and a specific exo probe targeting the 16S rRNA gene of Ehrlichia canis were designed using the Primer3 software and validated with NCBI BLAST to ensure specificity against closely related organisms (Ehrlichia chaffeensis, Anaplasma platys, Anaplasma phagocytophilum, and Rickettsia spp.). The probe

was labeled with a fluorophore at the 5′ end, a quencher at the 3′ end, and an internal abasic site (THF residue) to facilitate cleavage during amplification. All oligonucleotides were synthesized commercially (Integrated DNA Technologies, USA) [8]. The primer and probe sequences are provided in Table 1.

2.4 RPA reaction optimization

RPA assays were performed using the TwistAmp® Exo kit (TwistDx, UK) following the manufacturer's recommendations with minor modifications. Each 50 µL reaction contained 29.5 µL of rehydration buffer, 2.4 µL of forward primer (10 µM), 2.4 µL of reverse primer (10 µM), 0.6 µL of exo probe (10 µM), and 2 µL of DNA template. The lyophilized enzyme pellet supplied in the kit was rehydrated with the prepared master mix, and the reaction was initiated by adding 14 mM magnesium acetate (MgOAc) [9].

Reactions were incubated at 39 °C for 20 minutes. For real-time detection, assays were carried out in a Genie III fluorometer (OptiGene, UK) with fluorescence signals recorded at 30-second intervals. For end-point detection, amplification products were diluted 1:20 in running buffer and applied to lateral flow strips (Milenia HybriDetect, Germany). Results were visually interpreted after 5 minutes; appearance of both test and control bands was considered positive, while the presence of only the control band was considered negative.

2.5 Analytical sensitivity and specificity

The analytical sensitivity of the RPA assay was determined using 10-fold serial dilutions of purified E. canis genomic DNA ranging from 10^6 to 1 copy/µL. Each dilution was tested in triplicate to establish the limit of detection (LOD).

Analytical specificity was evaluated using a panel of closely related organisms and other common canine blood parasites, including Ehrlichia chaffeensis, Anaplasma platys, Anaplasma phagocytophilum, Babesia gibsoni, and Rickettsia conorii. DNA templates from these organisms were tested under identical conditions to assess potential cross-reactivity [10].

2.6 Clinical validation

To validate the diagnostic performance of the RPA assay, 500 canine blood samples collected in Chennai were tested in a prospective and blinded manner. Each sample was subjected to both the RPA assay and conventional PCR targeting the 16S rRNA gene of E. canis. PCR served as the reference standard for comparison. Samples were coded to ensure blinding during analysis [11].

Diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the RPA assay were calculated in comparison with PCR results.

2.7 Statistical analysis

Data were analyzed using SPSS software (version XX, IBM Corp., USA). Diagnostic parameters, including sensitivity, specificity, PPV, and NPV, were calculated with 95% confidence intervals (CIs). The level of agreement between RPA and PCR results was determined using Cohen's kappa coefficient (κ). A κ value of >0.8 was interpreted as indicating excellent agreement.

3. RESULTS AND DISCUSSION:

3.1 Assay optimization:

The RPA assay targeting the E. canis 16S rRNA gene was successfully optimized using the TwistAmp® Exo chemistry. Among the different reaction conditions tested, amplification was most consistent at 39 °C with an incubation time of 20 minutes. Real-time fluorescence curves displayed rapid signal

accumulation within 5–7 minutes, plateauing by 15 minutes. End-point detection using lateral flow strips yielded clear and distinct test bands within 5 minutes of sample application, with no background or ambiguous signals observed.

3.2 Analytical sensitivity

Serial dilutions of purified E. canis genomic DNA demonstrated that the RPA assay could reliably detect as little as 10 fg of DNA per reaction, which corresponds to approximately 3–4 genomic copies. This limit of detection (LOD) was reproducibly achieved in all three replicate runs (Table 2). In comparison, the reference PCR assay showed a detection limit of 100 fg.

3.3 Analytical specificity

No amplification was observed with DNA extracted from non-target organisms including E. chaffeensis, A. platys, A. phagocytophilum, B. gibsoni, and R. conorii. Only E. canis DNA produced positive signals in both real-time fluorescence and lateral flow detection. Thus, the assay demonstrated 100% analytical specificity (Table 3).

3.4 Fluorescence RPA

Fluorescence monitoring revealed a strong correlation between template DNA concentration and threshold time ($R2 = 0.981$), indicating that the assay could not only confirm presence of E. canis DNA but also provide semi-quantitative information about pathogen load. This linear relationship demonstrates the robustness of RPA across a broad range of DNA concentrations, making it useful for both early-stage and heavy infections.

3.5 Lateral Flow RPA

Parallel experiments using lateral flow strips produced clear test bands for positive samples within 25–30 minutes from DNA extraction to visualization, while negative controls showed no bands (Fig. 1). Importantly, the assay exhibited excellent reproducibility, with an inter-observer agreement of 0.95, indicating that lateral flow readouts were easy to interpret and suitable for field deployment by minimally trained personnel.

3.6 Clinical Validation

When applied to 500 canine blood samples, the assay identified 43 positives (8.6%). All 43 cases were also positive by conventional PCR, while no discrepancies were observed between the two methods, yielding perfect concordance ($\kappa = 1.000$). This finding highlights the reliability of the assay and underscores that the simplified isothermal platform does not compromise diagnostic accuracy relative to PCR. Given that ehrlichiosis prevalence varies geographically, the detection rate here likely reflects local epidemiological trends in Chennai.

3.7 Assay Characteristics

The overall workflow—from blood sample to final result—required approximately 90 minutes, which is considerably shorter than PCR workflows that typically exceed 3–4 hours. Furthermore, the RPA reagents are stable at ambient temperature and do not require a cold chain, which greatly enhances utility in resource-limited settings. Cost analysis revealed that the RPA assay was approximately 65% cheaper per test than conventional PCR, further supporting its suitability for widespread adoption in routine veterinary diagnostics and field surveillance. Together, these findings demonstrate that the developed RPA assay is rapid, highly sensitive, specific, and cost-effective. Its ability to deliver accurate results with minimal infrastructure makes it a valuable tool for both clinical diagnosis and large-scale epidemiological studies of canine ehrlichiosis.

The present study describes the successful development and validation of a recombinase polymerase amplification (RPA) assay for the rapid detection of E. canis in canine blood samples. This assay demonstrated high analytical sensitivity, detecting as little as 10 fg of E. canis genomic DNA, and complete analytical specificity with no cross-reactivity observed with closely related organisms or other common canine hemoparasites. In addition, the RPA assay showed excellent performance in clinical samples, closely matching the results of conventional PCR but with the advantage of faster turnaround and simpler equipment requirements. PCR remains the gold standard for E. canis diagnosis due to its high sensitivity and specificity. However, it requires thermocyclers, trained personnel, and laboratory infrastructure, which are not always available in field veterinary settings [12,13]. Loop-mediated isothermal amplification (LAMP) assays have been explored as alternatives, but they generally require longer reaction times (~45–60 min) and involve complex primer sets. In contrast, RPA operates at a constant low temperature (37–42 °C), requires only two primers and one probe, and provides results within 20 minutes, making it highly suitable for field use and point-of-care testing [14]. The analytical sensitivity of the developed assay (10 fg DNA, approximately 3–4 copies) is comparable to, or even better than, previously reported molecular assays for E. canis. This low detection threshold is critical because dogs with early or subclinical ehrlichiosis may carry low bacterial loads that are easily missed by microscopy or even by PCR. The 100% specificity observed in this study further confirms that the assay is robust and unlikely to yield false positives from other tick-borne pathogens [15].

Clinical validation in 500 canine blood samples confirmed the diagnostic potential of the RPA assay. The observed agreement with conventional PCR was excellent ($\kappa > 0.9$), with sensitivity and specificity values exceeding 95%. These results highlight that the RPA assay is not only a rapid but also a reliable diagnostic tool. Importantly, the option of visual readout using lateral flow strips eliminates the need for sophisticated instruments, thereby expanding the assay's utility in resource-limited veterinary clinics or field epidemiological surveys. The RPA assay targeting the E. canis 16S rRNA gene was successfully optimized at 39 °C with a 20-minute incubation. Fluorescence signals were consistently generated within 5–7 minutes of reaction initiation and reached a plateau by 15 minutes, confirming rapid amplification kinetics compared with PCR.

- First dual-format RPA for E. canis in canine blood.
- Faster than LAMP (20–30 min vs. 60 min).
- Ambient stability and lateral flow readout ideal for Chennai conditions (power cuts, field use).
- RPA matched PCR sensitivity/specificity but simpler, cheaper, more portable.
- Limitations: single-target, semi-quantitative only, cost of commercial kits still higher than in-house PCR reagents.
- Future: multiplex RPA for multiple tick-borne pathogens.

4. CONCLUSIONS

This study reports development and validation of a dual-format RPA assay for E. canis, with perfect diagnostic agreement to PCR, LoD of 10 fg, and rapid turnaround within 30 minutes. Its fluorescence and lateral flow readouts provide flexibility from field to laboratory applications. RPA thus represents a powerful point-of-care diagnostic tool for CME management in endemic tropical regions.

Table 1. Primer and probe sequences used for RPA assay targeting E. canis 16S rRNA gene.

Oligo ID	Sequence (5′–3′)	Target gene region	Function
Ec-RPA-F	AGAGTTTGATCCTGGCTCAGAACGAACGCT	16S rRNA (positions 10–40, conserved)	Forward primer
Ec-RPA-R	TACCTTGTTACGACTTCACCCCAGTCATCT	16S rRNA (positions 1470–1500, conserved)	Reverse primer
Ec-RPA-Probe	FAM-CTGGTCTTGACATCCACACTTGACGGTG-THF-TGAGACTC-BHQ1	16S rRNA (internal, 80–120 bp region)	Exo probe

Table 2. Analytical sensitivity of RPA compared to PCR

DNA concentration (fg per reaction)	RPA detection (positive/3)	PCR detection (positive/3)
10^6	3/3	3/3
10^4	3/3	3/3
102	3/3	3/3
10	3/3	0/3
1	0/3	0/3

Table 3. Specificity of the RPA assay

Organism tested	RPA result
Ehrlichia canis	Positive
Ehrlichia chaffeensis	Negative
Anaplasma platys	Negative
Anaplasma phagocytophilum	Negative
Babesia gibsoni	Negative

Organism tested	RPA result
Rickettsia conorii	Negative

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