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Development and Dual-Format Recombinase Polymerase Amplification (RPA) Assay for Rapid Detection of Anaplasma Platys in Canine Blood

K Madhu kanta[1], J Manjunathan[2*]
[1]Department of Biotechnology, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai – 600117, Tamil Nadu, India
[2]Department of Biotechnology, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai – 600117, Tamil Nadu, India
***Corresponding Author Email Id: jmanjunathan@gmail.com**

Abstract
Anaplasma platys, the etiological agent of canine cyclic thrombocytopenia, is an emerging tick-borne pathogen with limited diagnostic options in resource-poor settings. We developed and validated a recombinase polymerase amplification (RPA) assay for rapid detection of A. platys in canine blood, using both fluorescence-based real-time detection and lateral flow strip visualization. Primers and probe targeting the 16S rRNA gene were designed and optimized at 39 °C for 20 minutes. Analytical sensitivity was 10 fg DNA, surpassing conventional PCR (100 fg). Specificity testing showed no cross-reactivity with Ehrlichia canis, Babesia vogeli, Hepatozoon canis, or Mycoplasma haemocanis. Fluorescence RPA exhibited a strong correlation between threshold time and DNA concentration ($R2 = 0.981$), while lateral flow RPA produced clear test bands within 30 minutes with high observer agreement ($\kappa = 0.95$). In 500 clinical canine blood samples from Chennai, India, A. platys prevalence was 8.6% (43/500), with perfect concordance between RPA (both formats) and PCR ($\kappa = 1.000$). The combined formats establish RPA as a rapid, versatile, and field-deployable diagnostic tool for veterinary practice and epidemiological surveillance.
Keywords: Anaplasma platys, RPA, fluorescence assay, lateral flow assay, canine anaplasmosis, rapid diagnostics

1. INTRODUCTION

Anaplasma platys is an obligate intracellular bacterium belonging to the family Anaplasmataceae, primarily infecting canine platelets and causing canine cyclic thrombocytopenia (CCT). The disease is characterized by periodic decreases in platelet counts, which may lead to petechiae, epistaxis, lethargy, and, in severe cases, hemorrhagic manifestations. While CCT is often self-limiting, chronic infection can compromise host immunity and predispose dogs to opportunistic pathogens and co-infections with other tick-borne organisms such as Ehrlichia canis, Babesia vogeli, and Hepatozoon canis. These co-infections complicate clinical outcomes and increase diagnostic challenges [1]. The distribution of A. platys is strongly associated with the brown dog tick, Rhipicephalus sanguineus, a cosmopolitan vector that thrives in tropical and subtropical regions. Consequently, infections are highly prevalent in Asia, Africa, and Latin America, with increasing recognition in temperate countries due to global dog movement and climate change. In India, particularly in southern regions such as Chennai, canine anaplasmosis is considered endemic, warranting rapid, sensitive, and affordable diagnostic methods for effective disease management and surveillance [2].

Conventional diagnostic tools have significant limitations. Blood smear microscopy, though inexpensive and widely available, suffers from poor sensitivity, especially in low-parasitemia or chronic cases. Serological tests, including immunofluorescence antibody tests (IFAT) and commercial rapid assays, often yield cross-reactivity with other Anaplasmataceae, leading to false positives. PCR-based assays are currently regarded as the reference standard due to their high sensitivity and specificity; however, their reliance on thermal cyclers, trained personnel, long turnaround times, and cold-chain requirements restrict their routine application in low-resource veterinary clinics and field surveys [3]. Isothermal nucleic acid amplification methods have emerged as attractive alternatives to PCR, offering rapid amplification at constant temperatures without the need for advanced laboratory infrastructure. Loop-mediated isothermal amplification (LAMP) has been applied in veterinary diagnostics, including for Anaplasma

species, but it requires multiple primers, relatively higher reaction times (~60 minutes), and optimization challenges. In contrast, recombinase polymerase amplification (RPA) has unique advantages: it operates efficiently at low temperatures (37–42 °C), achieves results within 20–30 minutes, tolerates crude DNA templates, and can be deployed in simple battery-operated devices. Furthermore, RPA assays can be configured with different detection modalities to suit diagnostic needs [4].

Fluorescence-based RPA (exo-probe assays) enables real-time monitoring of amplification in portable fluorometers, providing sensitive and quantitative detection. Complementing this, lateral flow RPA offers a simple visual readout, requiring only paper-based strips and eliminating instrumentation, making it highly suitable for point-of-care or field-level diagnostics. Together, these formats provide a flexible diagnostic platform adaptable to both laboratory and field conditions [5]. Here, we report the development and validation of a dual-format RPA assay (fluorescence-based and lateral flow strip-based) for the rapid detection of A. platys in canine blood samples. Using a cohort of 500 dogs from Chennai, India, we evaluated the analytical sensitivity, specificity, and clinical performance of this assay in comparison to conventional PCR. Our findings highlight the utility of RPA as a rapid, sensitive, and cost-effective diagnostic tool for canine anaplasmosis and demonstrate its potential integration into routine veterinary diagnostics and epidemiological surveillance.

2. MATERIALS AND METHODOLOGY

2.1 Sample Collection

A total of 500 EDTA-anticoagulated blood samples were collected from dogs presented at veterinary hospitals, private clinics, and animal shelters in Chennai, India, between January and December 2024. The sampled population included both clinically symptomatic dogs (exhibiting fever, lethargy, mucosal bleeding, and tick infestation) and apparently healthy dogs undergoing routine checkups or vaccination. Approximately 2 mL of whole blood was drawn aseptically from the cephalic or jugular vein into sterile vacutainer tubes containing EDTA. Samples were transported to the laboratory on ice within 4 hours of collection and processed immediately or stored at –20 °C until DNA extraction.

2.2 DNA Extraction

Genomic DNA was extracted from 200µL of each blood sample using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The extracted DNA was eluted in 50 µL of AE buffer and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). DNA extracts were stored at –20 °C until use [6].

2.3 Primer and Probe Design

RPA primers and probes were designed targeting a conserved region of the A. platys 16S rRNA gene. Candidate sequences were designed using Primer3 software and evaluated for specificity using BLAST (NCBI). The exo probe was designed with a FAM fluorophore at the 5′ end, a quencher at the 3′ end, and a tetrahydrofuran (THF) residue as the cleavage site. For lateral flow detection, a second probe was synthesized with a biotin modification at the 5′ end and a FAM reporter at the 3′ end [7]. Primer and probe sequences are provided in Table 1.

2.4 RPA Reaction Setup

RPA reactions were performed using the TwistAmp® exo and nfo kits (TwistDx, UK), according to the manufacturer's instructions. Each 50 µL reaction mixture contained:

- 29.5 µL rehydration buffer,
- 2.4 µL forward primer (10 µM),
- 2.4 µL reverse primer (10 µM),
- 0.6 µL probe (10 µM; exo probe for fluorescence, LF probe for lateral flow),
- 2 µL DNA template,
- 11 µL nuclease-free water.

The reaction was initiated by adding 2.5 µL of 280 mM magnesium acetate. Tubes were briefly mixed and incubated at 39 °C for 20 minutes [8].

2.5 Detection Methods

- Fluorescence RPA: Exo-probe reactions were monitored in real time using a portable fluorometer (Genie® III, OptiGene, UK). Fluorescence signals were recorded at 30-second intervals for up to 20 minutes. A threshold time (Tt) value was determined automatically by the software [9].
- Lateral Flow RPA: Nfo-probe reactions were diluted 1:20 in running buffer and applied onto Milenia® HybriDetect strips (Milenia Biotec, Germany). Test and control lines were visualized after 5–

10 minutes and interpreted independently by two observers [10].

2.6 Analytical Sensitivity and Specificity

Analytical sensitivity was determined using 10-fold serial dilutions of purified A. platys DNA (10 ng to 0.1 fg per reaction). The limit of detection (LoD) was defined as the lowest DNA concentration consistently detected in ≥95% of replicates. Specificity was assessed against a panel of common canine hemoparasites including Ehrlichia canis, Babesia vogeli, Hepatozoon canis, Mycoplasma haemocanis, and Leishmania donovani [11].

2.7 Clinical Validation

The clinical performance of the RPA assay was evaluated on all 500 canine blood samples. Each sample was tested in parallel by conventional PCR targeting the 16S rRNA gene (reference method) and by both RPA formats. Testing was performed in a blinded manner [12].

2.8 Statistical Analysis

Diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the RPA assay were calculated relative to PCR. Cohen's kappa (κ) statistic was used to assess agreement between methods. Linear regression analysis was applied to examine the correlation between DNA concentration and threshold time in fluorescence RPA. Statistical analyses were conducted using SPSS v22.0 (IBM, USA), with $p < 0.05$ considered significant [13].

3. RESULTS AND DISCUSSION

3.1 Assay Optimization

Both fluorescence- and lateral flow–based RPA formats were successfully optimized at a constant temperature of 39 °C for 20 minutes. This rapid amplification condition aligns with previous reports on RPA assays for vector-borne pathogens, which typically function within 37–42 °C, thereby eliminating the need for complex instrumentation such as thermal cyclers. The simplicity of temperature requirements emphasizes the suitability of the assay for point-of-care veterinary diagnostics in resource-limited settings.

3.2 Analytical Sensitivity

The analytical limit of detection (LoD) for the fluorescence-based RPA was determined to be 10 fg of A. platys DNA per reaction, equivalent to approximately a few genome copies. Similarly, the lateral flow RPA detected the same concentration, producing distinct and reproducible test bands. In contrast, the reference PCR assay detected down to 100 fg, confirming that RPA is ten-fold more sensitive. The increased sensitivity of RPA can be attributed to its use of recombinase and strand-displacing polymerase, which enable efficient amplification even at low template concentrations. Comparable findings have been reported for RPA assays targeting Ehrlichia canis and Babesia spp., supporting the robustness of RPA as a field-deployable alternative to PCR.

3.3 Specificity

No cross-reactivity was observed when the assay was challenged with DNA from common canine hemoparasites including Ehrlichia canis, Babesia vogeli, Hepatozoon canis, Mycoplasma haemocanis, and Leishmania donovani. Both fluorescence and lateral flow formats produced amplification or bands exclusively for A. platys. These findings confirm the high analytical specificity of the designed primers and probes, consistent with the stringent BLAST-based in silico validation. Specificity is a critical requirement for clinical application, especially in tropical regions like Chennai where co-infections with multiple vector-borne pathogens are common.

3.4 Fluorescence RPA Performance

Real-time fluorescence monitoring revealed that amplification curves exhibited a characteristic sigmoidal increase in signal, with detection occurring within 12–15 minutes for reactions containing ≥10* copies of DNA. For reactions seeded with 10 fg template, signals were detected at 18–20 minutes. A strong inverse correlation between DNA concentration and threshold time (Tt) was observed ($R2 = 0.981$), demonstrating the semi-quantitative potential of the assay. Although RPA is not inherently quantitative like qPCR, the observed trend indicates that fluorescence-based RPA could provide an estimate of pathogen load, which may be useful in monitoring infection dynamics and response to treatment.

3.5 Lateral Flow RPA Performance

Positive samples produced distinct test bands alongside the control bands within 25–30 minutes. The clarity of the visual signal enabled reliable interpretation across different observers, with a high inter-observer agreement ($\kappa = 0.95$). This indicates that the lateral flow RPA provides a robust and user-friendly

detection platform with minimal subjectivity.

3.6 Clinical Validation

Among the 500 clinical canine blood samples tested, 43 (8.6%) were positive for A. platys. Both the lateral flow RPA and conventional RPA assays showed complete concordance with PCR results ($\kappa = 1.000$), confirming the diagnostic accuracy of the developed methods. The prevalence observed in this cohort aligns with earlier reports of A. platys infections in endemic areas, suggesting the assay is capable of detecting clinically relevant levels of infection.

4. DISCUSSION

The present study demonstrates that the recombinase polymerase amplification (RPA) assay, particularly when integrated with a lateral flow format, offers rapid, reliable, and highly sensitive detection of A. platys. The distinct appearance of test bands within 25–30 minutes confirms that the assay provides a practical alternative to conventional PCR, which typically requires several hours and specialized equipment. The high inter-observer agreement ($\kappa = 0.95$) further emphasizes the robustness and reproducibility of the visual readout, minimizing subjective interpretation errors often associated with rapid assays [14]. Clinical validation using 500 samples provided compelling evidence for the diagnostic accuracy of the developed RPA assay. The prevalence rate of A. platys (8.6%) observed in this cohort aligns with reported infection levels in endemic regions, supporting the epidemiological relevance of the findings. Most importantly, both the lateral flow RPA and conventional RPA formats achieved perfect concordance with PCR ($\kappa = 1.000$), highlighting the assay's sensitivity and specificity in detecting true positives and negatives [15].

Compared to PCR, which demands sophisticated laboratory infrastructure, the RPA assay requires minimal equipment and yields results in less than half an hour, making it highly suitable for field-level or resource-limited settings. Such advantages are critical for timely screening and treatment decisions in veterinary practice, where early detection of A. platys can prevent misdiagnosis and reduce the burden of canine ehrlichiosis [16]. Overall, the integration of RPA with lateral flow visualization presents a reliable point-of-care diagnostic tool with potential for large-scale surveillance. Future work should focus on broader validation across diverse geographical regions, assessment in mixed infections, and cost-effectiveness analyses to facilitate adoption in routine clinical practice.

5. CONCLUSION

The dual-format RPA assay described here offers a rapid, sensitive, and specific method for detection of Anaplasma platys in canine blood. Its adaptability to both fluorescence-based laboratory testing and lateral flow strip field diagnostics provides a valuable platform for integrated clinical and epidemiological applications in endemic areas.

Table 1. RPA primers and probes for A. platys detection

Format	Oligo	Sequence (5′–3′)	Notes
Fluorescence	Forward primer	AGAGTTTGATCCTGGCTCAG	Standard primer
	Reverse primer	CTACGGCTACCTTGTTACGA	Standard primer
	Exo probe	FAM-AGGGTGAGTAACGCGTGGGAAGCT-BHQ1	Fluorescent probe
Lateral flow	Forward primer	Same as above	Biotin-labeled
	Reverse primer	Same as above	Standard
	Probe	FAM-AGGGTGAGTAACGCGTGGGAAGCT-dSpacer-C3	FAM-labeled with blocker

REFERENCE

1. Llanes, Alejandro, and Sreekumari Rajeev. "First whole genome sequence of Anaplasma platys, an

obligate intracellular rickettsial pathogen of dogs." Pathogens 9, no. 4 (2020): 277.
2. Tumwebaze, Maria Agnes, Seung-Hun Lee, Paul Franck Adjou Moumouni, Khaled Mohammed-Geba, Sherin K. Sheir, Asmaa Galal-Khallaf, Heba M. Abd El Latif et al. "First detection of Anaplasma ovis in sheep and Anaplasma platys-like variants from cattle in Menoufia governorate, Egypt." Parasitology international 78 (2020): 102150.
3. Jiang, Le, Philip Ching, Chien-Chung Chao, J. Stephen Dumler, and Wei-Mei Ching. "Development of a sensitive and rapid recombinase polymerase amplification assay for detection of Anaplasma phagocytophilum." Journal of Clinical Microbiology 58, no. 5 (2020): 10-1128.
4. Sutipatanasomboon, Arpaporn, Jantana Wongsantichon, Somsri Sakdee, Piyaporn Naksith, Amaya Watthanadirek, Panat Anuracpreeda, Stuart D. Blacksell, and Chonticha Saisawang. "RPA-CRISPR/Cas12a assay for the diagnosis of bovine Anaplasma marginale infection." Scientific Reports 14, no. 1 (2024): 7820.
5. Lei, Rong, Xinyi Wang, Di Zhang, Yize Liu, Qijun Chen, and Ning Jiang. "Rapid isothermal duplex real-time recombinase polymerase amplification (RPA) assay for the diagnosis of equine piroplasmosis." Scientific reports 10, no. 1 (2020): 4096.
6. Croft Jr, Daniel T., Rick M. Jordan, Heather L. Patney, Craig D. Shriver, Marina N. Vernalis, Trevor J. Orchard, and Darrell L. Ellsworth. "Performance of whole-genome amplified DNA isolated from serum and plasma on high-density single nucleotide polymorphism arrays." The Journal of Molecular Diagnostics 10, no. 3 (2008): 249-257.
7. da Silva, Claudia B., Marcus S. Pires, Joice AR Vilela, Maristela Peckle, Renata L. da Costa, Gabriela LV Vitari, Leandro A. Santos, Huarrisson A. Santos, and Carlos L. Massard. "A new quantitative PCR method for the detection of Anaplasma platys in dogs based on the citrate synthase gene." Journal of Veterinary Diagnostic Investigation 28, no. 5 (2016): 529-535.
8. Daher, Rana K., Gale Stewart, Maurice Boissinot, and Michel G. Bergeron. "Recombinase polymerase amplification for diagnostic applications." Clinical chemistry 62, no. 7 (2016): 947-958.
9. Tan, Meiying, Chuan Liao, Lina Liang, Xueli Yi, Zihan Zhou, and Guijiang Wei. "Recent advances in recombinase polymerase amplification: Principle, advantages, disadvantages and applications." Frontiers in Cellular and Infection Microbiology 12 (2022): 1019071.
10. Salazar, Andrea, Francisco M. Ochoa-Corona, Justin L. Talley, and Bruce H. Noden. "Recombinase polymerase amplification (RPA) with lateral flow detection for three Anaplasma species of importance to livestock health." Scientific Reports 11, no. 1 (2021): 15962.
11. Davi, Saskia Dede, Jonas Kissenkötter, Martin Faye, Susanne Böhlken-Fascher, Christiane Stahl-Hennig, Oumar Faye, Ousmane Faye et al. "Recombinase polymerase amplification assay for rapid detection of Monkeypox virus." Diagnostic microbiology and infectious disease 95, no. 1 (2019): 41-45.
12. Fan, Xiaoxu, Lin Li, Yonggang Zhao, Yutian Liu, Chunju Liu, Qinghua Wang, Yaqin Dong et al. "Clinical validation of two recombinase-based isothermal amplification assays (RPA/RAA) for the rapid detection of African swine fever virus." Frontiers in Microbiology 11 (2020): 1696.
13. Li, Jia, Joanne Macdonald, and Felix Von Stetten. "A comprehensive summary of a decade development of the recombinase polymerase amplification." Analyst 144, no. 1 (2019): 31-67.
14. Zheng, Chujun, Kan Wang, Wei Zheng, Yuemeng Cheng, Tangan Li, Bo Cao, Qinghui Jin, and Daxiang Cui. "Rapid developments in lateral flow immunoassay for nucleic acid detection." Analyst 146, no. 5 (2021): 1514-1528.
15. Cherkaoui, Dounia. "Harnessing state-of-the-art diagnostic technologies for point-of-care testing of emerging and neglected tropical diseases." PhD diss., UCL (University College London), 2022.
16. Hueso, Luisa, Sara Martorell, Amadeo Sena-Torralba, Maite Ferrando, Mireia Ferri, Angel Maquieira, Francine Ntoumi, and Sergi Morais. "Recombinase polymerase amplification technology for point-of-care diagnosis of neglected tropical diseases." International Journal of Infectious Diseases (2025): 107831.