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Original article

Development of a rapid and highly accurate diagnostic test for detecting *Theileria equi* in horses using recombinase polymerase amplification (RPA) combined with nucleic acid lateral flow immunoassay

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Abstract

Equine piroplasmosis (EP) is a protozoan disease in horses, mainly caused by *Theileria equi,* and is associated with non-specific signs such as fever, anemia, and edema. The lack of specific clinical signs, coupled with the high prevalence of carrier horses, highlights the critical need for a rapid and highly accurate diagnostic test for the detection of *Theileria equi*. Existing methods, including molecular, serological, and microscopic techniques, require specialized equipment, skilled personnel, and considerable time.

The current study aims to develop a rapid and accurate assay for the detection of *Theileria equi* in horses by using pre-labeled DNA in combination with recombinase polymerase amplification (RPA) and a lateral flow device (LFD). The proposed assay was evaluated in terms of time efficiency, sensitivity and specificity. Of the 30 samples tested, 23 were positive for *Theileria equi*, with no false positives observed. Moreover, the assay demonstrated a detection limit as low as 5 ng per microliter of input DNA, and the entire process from sample preparation to result interpretation was completed within 25 minutes without the need for specialized equipment. The findings demonstrate that the proposed assay exhibits 100% specificity, high sensitivity, and strong potential for clinical application. However, further studies are required to optimize the assay for direct pathogen detection from samples, eliminating the need for DNA extraction.

Keywords: pre-labelled amplicon, recombinase polymerase amplification (RPA), *Theileria equi*

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Introduction

Equine piroplasmosis (EP) is a tick-borne disease caused by haemoprotozoan parasites *Theileria equi* and *Babesia caballi* (Sumbria and Singla, 2015). EP can present as either an acute or chronic disease in horses, characterized by fever, lethargy, anemia, jaundice, and reduced appetite. Affected horses may also show weight loss, edema, colic, exercise intolerance, and in severe cases hemoglobinuria or death. EP is primarily transmitted by ticks belonging to different genera, including Hyalomma, Rhipicephalus and Dermacentor (Onyiche et al. 2019). Recognize of the type of tick in the infected area is crucial to control and minimize the infection. Several other factors such as season, weather, tick reproductive ability and availability of the host also influence transmission of this disease (Grech-Angelini et al. 2020). Because of the genetic variation and absence of precise diagnostic methods, diagnosis of EP is challenging and needs further research to determine a fast and accurate method. There are various detection methods such as blood smear for earlier identification of the parasite in the RBCs. Also, serological methods such as ELISA, Western blot, immunofluorescence assay and complement fixation test (Rothschild 2013, Wise et al. 2013, Facile et al. 2025). Additionally, molecular detection of the parasite enables the diagnosis of the disease at its early stage, both in carriers and during active infection. (Onyiche et al. 2019). The genetic diversity and the absence of a sensitive and rapid assay that can easily be used to detect the piropolasms that caused EP are major challenges (Nehra et al. 2024). These challenges were behind the recent research to find a fast and accurate method for EP diagnosis in horses. In the current study a rapid test was based on the detection of nucleic acid was developed. The assay was based on combined RPA, an LFD and a labelled primer for rapid detection of the *Theileria equi* within a short time and with no need for instruments or devices.

Materials and Methods

Sample collection

During 2024, 30 blood samples were collected from horses at the Iraqi Equestrian Club, Baghdad, Iraq. DNA was extracted using a blood DNA extraction kit (Qiagen) and kept at -80°C until the next step.

Designing and synthesis of RPA oligonucleotide

A conservative sequence of the erythrocyte merozoite antigen 1 (EMA-1) gene was selected as a target gene for the detection of *Theileria equi*. According to TwistDx™ instructions (https://www.twistdx.co.uk/wp-content/uploads/2021/04/twistamp-assay-design-manual), an RPA primer was been designed (Table 1). Synthesis of the primer was performed by Macrogen® (Korea). The forward primer was labeled with 6-FAM and the reverse primer was fused with Biotin.

Synthesis of EMA-1 gene

A conservative sequence of the EMA-1 gene was synthesized using Macrogen® (Korea). The synthetized EMA-1 was cloned in pMG vector (Macrogen®) in order to use it as a positive control and also to optimize the specificity and sensitivity of the assay.

RPA reaction and pre-labelling of the amplicon

A TwistAmp® Basic kit (TwistDx®, UK) was used to performed RPA which was used to amplify the DNA fragment of the target gene. The RPA reaction consisted of 2.5 ul of FAM labeled primer, the same volume of biotin labeled reverse primer, 4 ul of template (the extracted DNA from the sample) and 29.5 of resuspension buffer (provided with the kit) and the volume of the reaction was then adjusted to 47.5 ul. The whole reaction was then added to a lyophilized enzyme mixture provided by the manufacturer and the reaction was started by adding 2.5 ul of 280 mM of magnesium chloride and incubated at 37°C for 30 minutes. The resultant amplicon had FAM at the five end and biotin at the three end.

Reference standard PCR for *T. equi*

The diagnostic performance of the tested assay was validated using conventional PCR targeting the erythrocyte merozoite antigen-1 (EMA-1) gene of T. equi as the reference standard. The forward primer (5′-GAGG GCTATGCAGTTGAGAAGGTCAAGGAAGGC GA-3′) and reverse primer (5′-CTCGAGCTCGAC CCACTTATCACCGTCCTT-3′) were used to amplify a partial sequence of the EMA-1 gene. PCR reactions were performed in a total volume of 20 μL, containing 3 μL of extracted DNA template, 0.2 μM of each primer, 10 μL of 2× Taq Master Mix (Tinzyme, China), and 6 μL of nuclease-free water. Amplification was carried out in a thermal cycler under standard cycling conditions, with an annealing temperature of 58°C.

Lateral flow assay

Readout the RPA product was performed using a PCRD cassette (Abingdon Health®). According to the manufacturer is instruction 80μLof HybriDetect assay buffer was mixed with 20 ul of RPA reaction. Results of the test appear within 10-15 minutes, positive results show two lines on the cassette, negative shows one line and no line refers to invalid results.

Table 1. Primers used in the pre-labeled DNA assay for detection of *Theileria equi*.

Primer name	Sequence
TEF+P	5'-/6-FAM/GAGGGCTATGCAGTTGAGAAGGTCAAGGAAGGCGA
TER+P	CTCGAGCTCGACCCACTTATCACCGTCCTT/3Biotin/-3
EMA-1 fragment	GAGGGCTATGCAGTTGAGAAGGTCAAGGAAGGCGACTCCGTCATCAAGACCTTTGACTTGAAG GAACAAACCCCAAAGACTGTCGTCAGGCACATCAAGGACAACAAGCCATACGTCGTCATCGCCGTT GAGTCCGCCCTTCACCTCGTTCTCAAGAAGGACGGTGATAAGTGGGTCGAGCTCGAG

Experimental design

We developed a rapid, accurate and field test for detection of *Theileria equi* in horses. The assay depends on pre-labeled DNA and RPA in combination with lateral flow immunoassays. The efficiency of the assay was tested using three types of samples. In the test group, DNA was extracted from blood of horses clinically infected with *Theileria equi*. The second group was the control positive, in which the EMA-1 (target gene) was synthesized using gen art by (Macrogene®). The final group served as the negative control, consisting of animals that were clinically normal and showed no clinical signs of *T. equi* infection. Two labeled primers with FAM and biotin were used for amplification of the conservative sequence of the EMA-1 gene using RPA. The positive pre-labeled amplicon was read with PCRD.

Amplification of the EMA-1 using RT-RPA

The conservative sequence of the EMA-1 was amplified using RPA. The RPA that was used to produce the pre-labelled DNA included a forward primer labelled with FAM and a reverse primer labelled with biotin. The amplification reaction included a DNA template extracted from the positive and / or negative sample to Theileria equi, two labelled primers and the RPA enzyme mixture (Recombinase, polymerase and single -strand DNA binding protein), provided by TwistDx®, UK. The amplification was performed at room temperature without using any devices. The pre-labelled amplicon was passed on to the next step.

Results

Detection of EMA-1 gene in the tested sample

Detection of the EMA-1 gene in the tested samples was performed using lateral flow immunoassays. The pre-labeled amplicon from the positive sample to *Theileria equi* was readout using lateral flow immunoassays. Detection of the dual labelled amplification of the EMA-1 gene was performed using a PCRD cassette (Abingdon Health®) (Fig. 1). In the positive sample, anti FAM antibodies were bund to the FAM labelled end of the amplicon. Gold nanoparticle were then attached to the anti FAM antibody, which makes the amplicon visible and appears as a line in the PCRD cassette. Also, gold nanoparticles attach to anti-rabbit antibody and makes the control line visible in the PCRD cassette. Samples with 2 lines (sample and control line) were considered as positive to *Theileria equi* while samples with one line were considered as negative to *Theileria equi* (Fig. 2).

Evaluation of the established assay using clinical specimens

Efficiency of the established assay was performed by measuring the ability of the assay to detecting the target parasite in the clinical sample. Thirty blood samples were randomly collected from horses from the Iraqi Equestrian Club, Baghdad Iraq. The L-RPA-LFD identified 25 (83.3%) samples as positive to Theileria equi and 5 (16.7) as negative. 24 positive samples were verified with PCR. Also PCR verified 5 negative samples (17.6%). The accuracy of the assay was 100% for the negative samples and 96% for the positive samples. As a comparison with PCR, the L-RPA-LFD is a fast assay since it takes just 25 minutes to read out the results while PCR takes from 60 to 90 minutes to get the results. Furthermore, The L-RPA-LFD is performed at room temperature and need no any devices or skilled persons.

Specificity and Sensitivity of the L-RPA-LFD assay

To check the validity of the L-RPA-LFD assay for clinical uses, the specificity and sensitivity of the test were determined. Confirmed positive samples to *Anaplasma phagocytophilum* were used to check the specificity of the L-RPA-LFD assay. The results show there is no cross reactivity between primers and probe, and all Anaplasma positive samples were negative in the Theileria *equi*. Furthermore, the assay shows positive results for the positive control (synthetic EMA-1 gene) and negative results for the non-template control NTC (negative control) (Fig. 2). Genomic DNA extracted from the blood of horses was diluted from 20 ng to 5ng. The results show that the L-RPA-LFD

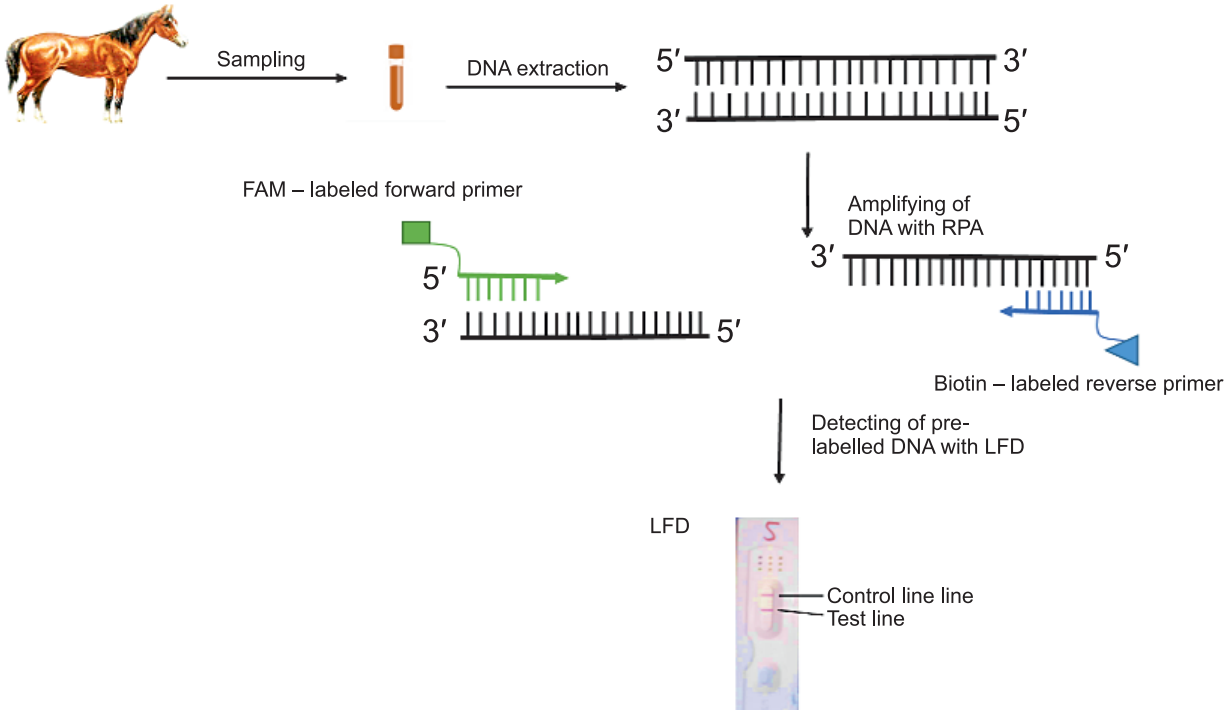
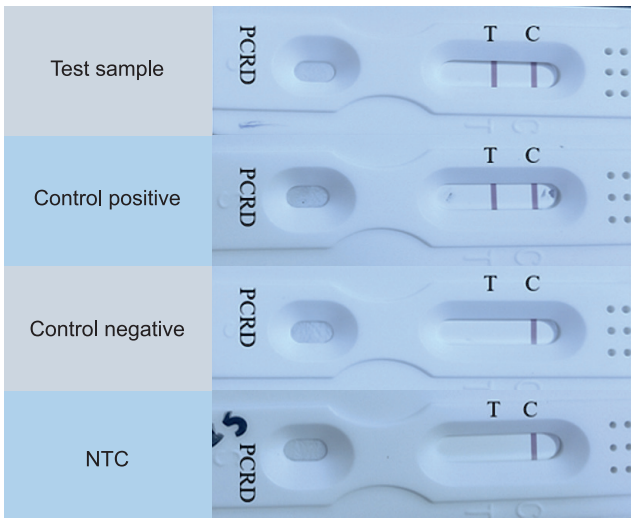


Fig. 1. RPA-LFD assay. DNA was extracted from blood of horses infected with *Theileria equi*. The forward primer was labeled with 6-FAM, while the reverse primer was fused with Biotin. Labelled amplicons were read using an LFD cassette (PCRD).

A



B

Sample type	Test line	Control line	Result
Tested sample	+	+	positive
Control positive	+	+	positive
Control negative	-	+	negative
NTC	-	+	negative

Fig. 2. RPA-LFD assay readout. A: LFD cassette represent. From top to bottom the first cassette represents the test sample, the second one represents the result of the positive control sample, the third represents control negative and the last represents the non template control (NTC). B: interpretation of the results, the cassette with two lines is considered as positive results while the cassette with one line is considered as negative.

assay is able to detect up to 5ng of the input DNA (Fig. 3). Ability of the assay to detect low concentrations of DNA indicates the sensitivity of the assay, which may be useful in case of low parasitemia and subclinical form of the disease.

Discussion

Equine piroplasmosis is a worldwide tick born disease infecting the equid family and is caused by *Theileria equi* and *Babesia caballi*. According to several studies *Theileria equi* is more common and more pathogenic than B. caballi (Mahmoud et al. 2016, Facile et al. 2025). Control and treatment of the disease requires efficient diagnostic methods. Blood smears

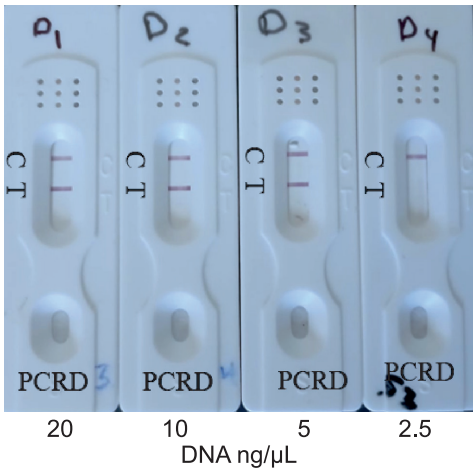


Fig. 3. Sensitivity of the RPA-LFD test. D1-D4 represent different concentrations of the input DNA. The results show that the RPA-LFD can detect up to 5 ng of the inputted DNA input.

are routinely used to detect the blood parasite, however, the parasite cannot be detected by this method during the latency period or in the chronic form of the disease. On the other hand, serological methods such as ELISA test, the complement fixation (CF) test and the indirect fluorescent antibody test (IFAT) are effectively used to diagnose of *Theileria equi*, but these tests need time and skilled persons and there some diagnostic limitation related to a high percentage of false positive (Santos et al. 2019). In general, the false positives of some serological tests are related to cross reactivity with antibodies from other infections. Molecular based detection including PCR and qPCR is a highly sensitive method. A rapid test based on the detection of nucleic acid is widely used nowadays (Jouglin et al. 2025). The ability to detect low parasitemia in *Theileria equi* infection, together with the time-consuming nature of performing assays and the ability to diagnose subclinical, carrier and chronic infections, are the main features that researchers and veterinarians are looking for in a rapid test (Lei et al. 2020, Tirosh et al. 2020, Mendoza et al. 2024). In the current study, a rapid test based on the detection of nucleic acids of *Theileria equi* was developed. RPA was used to amplify of the target sequence instead of PCR, the RPA reaction was performed at room temperature and without equipment. In this candidate test forward and reverse primers that were used in RPA were labeled with 6-FAM for the forward primer and for the reverse primer. The results of RPA amplification were read out using PCRD (nucleic acid lateral flow immunoassay based test). According to the results the developed assay is highly sensitive, specifics and rapid; requires no equipment and can be performed on the farm.

Conclusion

A rapid test based on the detection of nucleic acid is a good option for accurate diagnosis of an infectious agent. In this study, a combination between RPA with LFD was used to develop a new test for the detection of *Theileria equi* in horses. The results show the candidate test is highly specific and sensitive in the detectiom of low concentrations of DNA, it is a rapid test requiring no equipment. The test might be a particularly good option for detecting the parasite in case of subclinical and chronic forms of the disease.

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Author Declarations

Ethical approval

The research was approved by the Scientific Committee at the College of Veterinary Medicine, University of Al-Qadisiyah, under Order No. 53652, dated 15 July 2023.

Use of generative artificial intelligence

The authors confirm that no artificial intelligence (AI) tools were used during the design, analysis, or writing of this study.

Conflict of interest

The authors declare that there is no conflict of interest.

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