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On-site DNA Detection of Trypanosomatid Parasites and *Nosema ceranae* Through Alkaline Lysis Coupled to RPA/CRISPR/Cas12a System

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Citation

Carreira de Paula, J., Solano Parada, J., Rosel Miñarro, J.F., García Olmedo, P., Orantes, F.J., Osuna, A., de Pablos Torró, L.M. On-site DNA Detection of Trypanosomatid Parasites and *Nosema ceranae* Through Alkaline Lysis Coupled to RPA/CRISPR/Cas12a System. *J. Vis. Exp.* (221), e68874, doi:10.3791/68874 (2025).

Date Published

July 18, 2025

DOI

10.3791/68874

URL

jove.com/video/68874

Abstract

Early on-site detection of microscopic pathogens is essential for the effective management and health of honeybee colonies. Currently, the gold standard for molecular detection of bee pathogens is qPCR or RT-qPCR. Here, we present a rapid, sensitive, and cost-effective alternative for field-deployable DNA pathogen detection. This method combines direct, amplification-ready cell lysis of worker bee abdomens with recombinase polymerase amplification (RPA), CRISPR/Cas12a-mediated trans-cleavage of reporter probes, and detection via lateral flow assays. We successfully validated this protocol in honeybees infected with *Nosema ceranae* and *Lotmaria passim*. The described protocol can be performed using a simple heat block or at room temperature and is potentially applicable to any DNA-based pathogen or gut microbiota of the honeybee. The entire process takes approximately 180 min and achieves a sensitivity comparable to qPCR, detecting as few as 96 parasite copies/µL. In conclusion, this approach offers a promising tool for reliable and rapid field diagnosis of honeybee infections without the need for complex laboratory equipment, making it ideal for on-site colony surveillance.

Introduction

Bees are key species implicated in the pollination of plants and are essential for plant reproduction, biodiversity, and food security[1]. Their pollination services benefit a wide range of crops with high economic value, contributing an estimated $235–577 billion annually to global agriculture. However, the worldwide decline in bee populations has raised serious concerns due to its potential consequences for ecological stability and crop productivity, which have resulted in an estimated loss of $4.8–16.3 billion in heavily indebted poor countries[2]. The mass of bee disappearance involves multiple

interactions between several factors: pesticide application, habitat loss, climate change, and infections by pathogens, including viruses, bacteria, fungi, and protists.

Among these microorganisms, *Nosema* spp., a microsporidian fungus that disturbs the functioning of the gut, and trypanosomatid parasites — particularly *Lotmaria passim* — are widely distributed in honeybee colonies worldwide[3, 4]. Recent findings show that *L. passim* infections, even when asymptomatic, can downregulate immune-related genes and alter energy metabolism in honeybees, potentially compromising their resilience under additional stress[5]. Similarly, *Nosema* spp. infections reduce bee longevity, suppress immune function, and disrupt the colony's foraging dynamics, leading to reduced honey production and increased colony mortality. Moreover, coinfections involving both *Nosema* spp. and trypanosomatids may have synergistic effects, exacerbating physiological stress and weakening host defenses. These findings underscore the importance of monitoring multiple pathogens simultaneously.

Addressing these threats requires the development of rapid and accurate diagnostic tools, which in turn depend on a deeper understanding of the biology, transmission, and survival strategies of these pathogens[6]. The detection of these pathogens through traditional methods, including light microscopy and molecular amplification, remains reliable but faces certain limitations. Examples of qPCR, RTqPCR (requires cycling at different temperatures), and isothermal methods, such as Loop Mediated Amplification (LAMP, operates at constant temperature), have been developed for fungal, viral, bacterial, or protozoan parasites[6,7,8,9,10]. However, the implementation of these methods needs specialized equipment, together with trained personnel and well-equipped laboratory facilities, which restricts their practicality for field applications and large-scale monitoring. To address these issues, new molecular techniques have been developed — the amplification of DNA targets with recombinase polymerase amplification (RPA), together with CRISPR/Cas12a-based detection — a fast, specific, and sensitive approach. The method works isothermally and produces visual results that make it suitable for on-site or point-of-care diagnostics, according to Shao et al.[11] and Xiao et al.[12].

RPA is a simple and rapid DNA amplification method that operates at a constant temperature, typically between 37 °C and 42 °C. It relies on recombinase enzymes, single-stranded DNA-binding proteins, and a strand-displacing polymerase to amplify DNA without the need for thermal cycling. RPA is especially suitable for on-site testing and field diagnostics due to its speed, minimal equipment requirements, and compatibility with crude samples[11,12,13]. To enhance specificity, the RPA product can be detected using the Cas12a endonuclease, which also functions within the same temperature range. Cas12a binds to a CRISPR guide RNA (gRNA), a short RNA that guides Cas12a towards the amplified target sequence and binds to the complementary sequence in the DNA. Upon binding, Cas12a becomes activated and exhibits collateral (trans) cleavage activity, specifically against single-stranded DNA (ssDNA). This enables the use of ssDNA reporter molecules labeled with fluorophores or biotin, which can generate fluorescence or visual signals, including lateral flow strip readouts. Together, RPA and CRISPR/Cas12a form a powerful molecular diagnostic platform that is rapid, sensitive, and suitable for use in field or resource-limited settings[12].

To further enhance the practicality of this molecular diagnostic approach, we also implemented a simplified amplification-

ready cell lysis (ARCL) method based on sodium hydroxide (NaOH) lysis of bee abdomens[11,14], eliminating the need for extensive sample preparation and laboratory reagents. This rapid nucleic-acid extraction technique, combined with the isothermal amplification and CRISPR/Cas12a detection system, enables a truly field-deployable workflow that significantly reduces both time and cost. Importantly, the assay maintains the quality, high sensitivity, and specificity comparable to qPCR, the current gold standard for pathogen detection in bees.

Here, we successfully applied this methodology for the detection of *Nosema ceranae* and *L. passim* in honey bees (**Figure 1**). We compared this method to standard qPCR assays and validated its performance using naturally infected samples. Our findings show that this approach is a practical and reliable alternative for field-based monitoring of honeybee health, with strong potential for large-scale surveillance and early intervention efforts in beekeeping.

Protocol

All procedures were conducted in accordance with institutional and national guidelines for working with invertebrates and using the lowest number of bees needed for proof of concept. Honeybees were transported in specialized hive boxes with ventilation and appropriate temperature and stored in the lab at -20 °C until analysis.

1. HotSHOT alkaline cell lysis

1. Solution preparation
    1. HotSHOT Lysis buffer-preparation of 100 mL
        1. Weigh 99.993 mg of NaOH and 8.32 mg of EDTA, add 90 mL of distilled water, and mix. Adjust the pH to 12 and the volume to 100 mL; autoclave.
    2. 40 mM Tris-HCl, pH 5
        1. To 484.544 mg of Tris, add 90 mL of deionized water, adjust the pH to 5 with HCl, and bring the volume to 100 mL.
2. Lysis protocol
    1. Dissect the abdomen of the bee using a sterile scalpel and transfer the dissected tissue to a 1.5 mL microcentrifuge tube.
    NOTE: For pathogen detection, only the abdomens were used, and the remaining body parts were discarded.
    2. Add 400 µL of HotSHOT buffer, macerate the tissue using a disposable pestle, and further homogenize it by vortexing.
    NOTE: The pestle can be reused if decontaminated by incubating the material overnight in 0.1 N NaOH solution, then rinsed with double deionized water, followed by one autoclave cycle and subsequent drying in an incubator.
    3. Incubate the homogenate at 95 °C for 10 min to lyse the cells. After incubation, immediately place the tube on ice to cool.
    4. To neutralize the reaction, add an equal volume (400 µL) of 40 mM Tris-HCl, resulting in a final concentration of 20 mM Tris-HCl in the solution.
    NOTE: This lysis procedure is illustrated in **Figure 2**.

2. Isothermal amplification by RPA

NOTE: The design of the primers was made with the help of the eprimer3 software tool (https://www.bioinformatics.nl/cgi-

bin/emboss/eprimer3), although other primer designing tools could be used.

1. Preparation of working solutions
    1. RPA primer solutions
        1. Prepare **stock solutions** at a concentration of 100 µM according to the manufacturer's instructions. For example, reconstitute 10.0 nmol of lyophilized primer in 100 µL of Diethyl Pyrocarbonate (DEPC)-treated water and store at -20 °C. Prepare working solutions at a 10 µM concentration by diluting the stock 1:10; mix 90 µL of DEPC-treated water with 10 µL of the 100 µM stock solution in a 0.2 mL tube. Store these solutions at -20 °C.
2. RPA protocol
    1. Prepare the master mix according to the number of reactions to be performed. Include both a negative control (substituting ARCL with DEPC-treated water) and a positive control using pathogen DNA (in this example, DNA from cell culture *L. passim* promastigotes and bees positive for *Nosema* spp. (spores detected by microscopy)) (**Table 1**).
    NOTE: Always prepare the RPA master mix on ice and use filter tips to avoid contamination.
    2. Transfer an aliquot of 46.5 µL of the master mix into each 0.2 mL tube.
    3. Add magnesium acetate (MgOAc) and template DNA to the caps of the tubes, following the volumes specified in **Table 2**. Briefly centrifuge the tubes to mix the contents and initiate the RPA reaction.
    NOTE: For better reproducibility, briefly vortex and centrifuge all components before use. Positive and negative controls must be included in each batch to validate the reaction.
    4. Incubate the samples at 39 °C for 40 min for the RPA amplification reaction.
    NOTE: After incubation, RPA reactions can be directly used for downstream detection (e.g., CRISPR/Cas12a-based assays), described in section 3.

3. Pathogen DNA detection by CRISPR/Cas12a

NOTE: Detection after RPA can be performed in different ways. In this protocol, we will detail detection by fluorescence (FAM-TTTTTTTT-MGB) and immunochromatographic strips (Biotin-TTTTTTTT-FITC).

Perform all procedures in a clean, DNA-free area (e.g., dedicated PCR space). Clean work surfaces and pipettes with 0.5% bleach, followed by 70% ethanol. Always use filter tips and wear gloves. Handle DNA or amplified material in a separate area from the reagent preparation zone. Thaw all the reagents on ice and maintain cold conditions throughout preparation.

1. Preparation of working solutions
    1. crRNA
    NOTE: The designing of *Nosema ceranae* and *Lotmaria passim* crRNA was performed using the Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT) http://grna.ctegd.uga.edu/. For customized crRNA design, other software such as GPP sgRNA Designer (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) or CHOPCHOP (https://chopchop.cbu.uib.no/) may be used.

1. To prepare a 100 µM crRNA stock solution, reconstitute 10 nmol of each lyophilized crRNA with 100 µL of DEPC-treated water and store at -20 °C. To prepare a 1 µM working solution, perform a 1:100 dilution by mixing 1 µL of the 100 µM stock solution with 99 µL of DEPC-treated water in a 0.2 mL tube (**Figure 3**).
   NOTE: Both solutions should be stored at -20 °C.

2. Cas12a preparation
   NOTE: Cas12a can be provided at concentrations of either 1 µM or 100 µM.
   1. If supplied at 100 µM, dilute it to a 1 µM working solution before use by mixing 1 µL of the 100 µM Cas12a enzyme with 99 µL of the diluent provided in the kit (1:100 dilution, **Figure 3**).
      NOTE: The Cas12a enzyme concentration can be adjusted depending on the sample type. As the enzyme is stored in a glycerol-based diluent, it is important to ensure that the volume of enzyme added does not exceed 20% of the total reaction volume to avoid potential inhibitory effects.

3. Preparation of FAM and Biotin probe solutions
   NOTE: The FAM probe is provided in lyophilized form.
   1. To prepare a 100 µM stock solution, resuspend the probe by adding 1 µL of DEPC-treated water per 1 nmol of probe (e.g., for 102 nmol, add 102 µL of DEPC-treated water). Store the stock solution at -20 °C. For use in the detection mastermix, dilute the stock 1:10 (90 µL of DEPC-treated water with 10 µL of 100 µM FAM) to obtain a 10 µM working solution (**Figure 3**).

2. CRISPR/Cas12a detection protocol
   1. Fluorescence-based detection (FAM probe)
      1. In a 1.5 mL microcentrifuge tube, prepare the reaction mix as described in **Table 3**, excluding the amplicons.
      2. Mix thoroughly using a vortex and distribute the mixture into PCR tubes.
      3. Then, add 4 µL of RPA amplicon (described in step 2.2) to each tube.
      4. Incubate the reactions at 37 °C for 120 min in a thermocycler with fluorescence reading capability, recording fluorescence every minute.
      5. *Optional*: At the end of the incubation, transfer the reactions to 0.2 mL tubes and visualize using a gel documentation system for imaging.
   2. Detection using a Biotin probe in a lateral flow test
      1. In a 1.5 mL microcentrifuge tube, add the components shown in **Table 4**.
      2. Make the mix as described in section 3.2.1, mix, and distribute it into a PCR plate. Finally, add 4 µL of amplicon to the respective wells.
      3. Incubate for 120 min in a thermocycler (without fluorescence reading) or thermal block at 37 °C.
      4. Transfer the samples to 0.2 mL tubes and perform the detection with immunostrips. To do this, add 50 µL of running buffer and 10 µL of the reaction mixture into each tube and insert the strip directly into the tube. Incubate for 15 min

at room temperature and read the results after 10 min of immersion, as illustrated in **Figure 3**.

Representative Results

Here we have described a fast on-site method using alkaline lysis of worker bee abdomens coupled to RPA amplification and CRISPR/Cas12a-based detection by fluorescence and/or lateral flow approaches. The schema of the full workflow is represented in **Figure 3**, and the schema of the HotSHOT alkaline lysis of worker bee abdomens is in **Figure 4**. Representative results of the RPA/CRISPR/Cas12a assay are shown in **Figure 5**. To detect *L. passim* and *N. ceranae*, a thermocycler was used to quantify the relative fluorescence units (RFUs) using a FAM-labeled probe (**Figure 4A**). The RPA reactions were performed using 10 ng of DNA, and the fluorescence data were plotted in a graph (**Figure 4B**). At the final stage of the assay, samples in conical tubes were photographed using a gel imaging system (**Figure 4C**). For detection with a biotin-labeled probe, the samples were incubated in a thermoblock (**Figure 4A**); the detection solution was prepared according to the manufacturer's instructions and applied onto a lateral flow strip (**Figure 4B, C**). The method resulted in a successful detection of both honeybee pathogens.

To determine the efficiency of RPA reactions, we compared this method with standard qPCR using DNA purified from honeybees using the referenced kit (see the **Table of Materials**). A total of 32 bee samples were tested with primers targeting the *β-tubulin* gene of *L. passim* for both methods and then detected by CRISPR/Cas12a trans-cleavage of fluorescent probes. Notably, as a result, 12 samples were found to be positive by qPCR, while RPA/CRISPR/Cas12a detected *L. passim* in 16 samples (**Figure 5A**). This indicates that RPA reaction performance is more sensitive than PCR, as the gold-standard DNA amplification method.

Likewise, we determined the detection limit (LOD) for qPCR and RPA coupled to CRISPR/Cas12a detection for *L. passim* DNA as the pathogen model using serial dilutions of *L. passim* DNA ranging from 66 ng to 6 fg. qPCR detected as little as 6 pg, corresponding to approximately 96 parasites (**Figure 5B**). In comparison, the LOD for RPA corresponded to 96 parasites (**Figure 5C, D**).

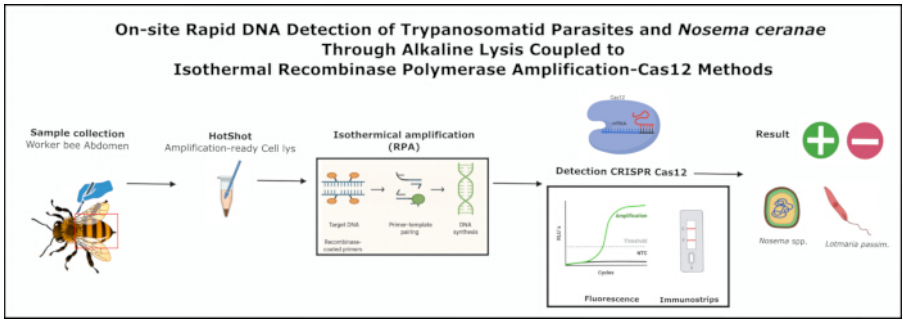

Figure 1: General workflow of the HotSHOT alkaline lysis coupled with RPA amplification and Cas12 detection methods in honeybees. Abbreviations: RPA = recombinase polymerase amplification; CRISPR = Clustered Regularly

Interspaced Short Palindromic Repeats; Cas = CRISPR-associated protein. Please click here to view a larger version of this figure.

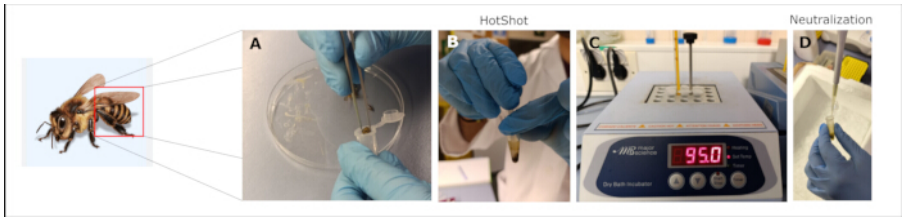

Figure 2: Processing of amplification-ready cell lysis with HotSHOT buffer. (**A**) Cut the honeybee abdomen with a scalpel and put it in a centrifuge tube; (**B**) with a pestle, make a maceration with 400 µL of HotShot; (**C**) incubate on a thermoblock at 95 °C for 10 min; and (**D**) neutralize with 400 µL of 40 mM Tris-HCl. Please click here to view a larger version of this figure.

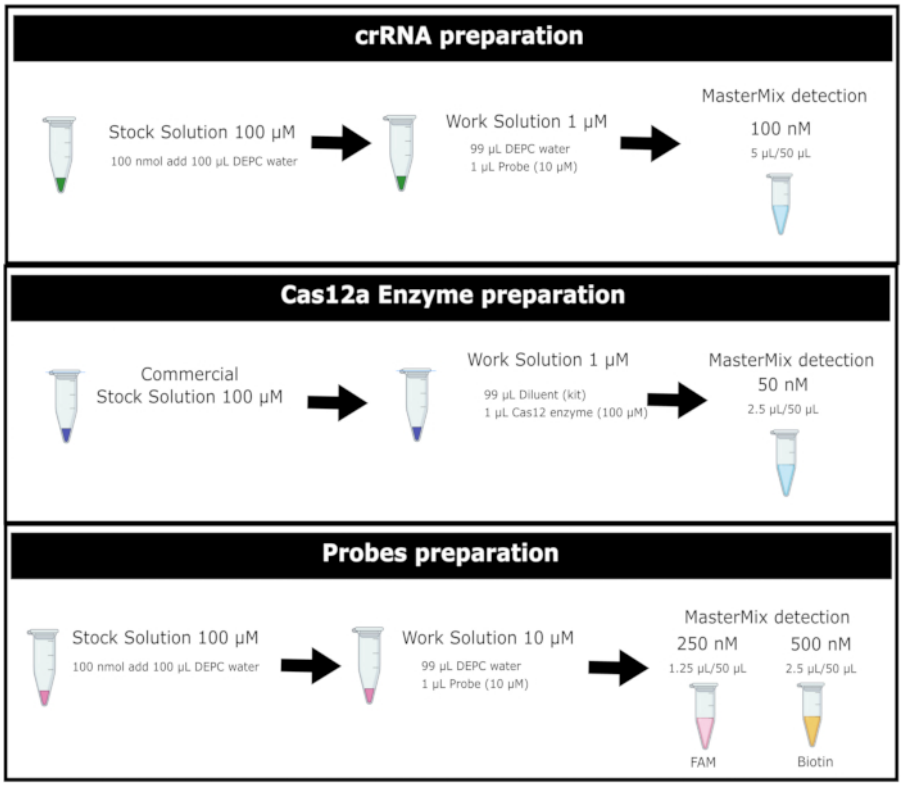

Figure 3: General workflow for the preparation of Cas12a enzyme and FAM- and Biotin-labeled reporter probe solutions. Abbreviations: DEPC-water = Diethyl Pyrocarbonate-treated water; crRNA= RNA CRISPR; Cas12 = type of CRISPR-associated protein. Please click here to view a larger version of this figure.

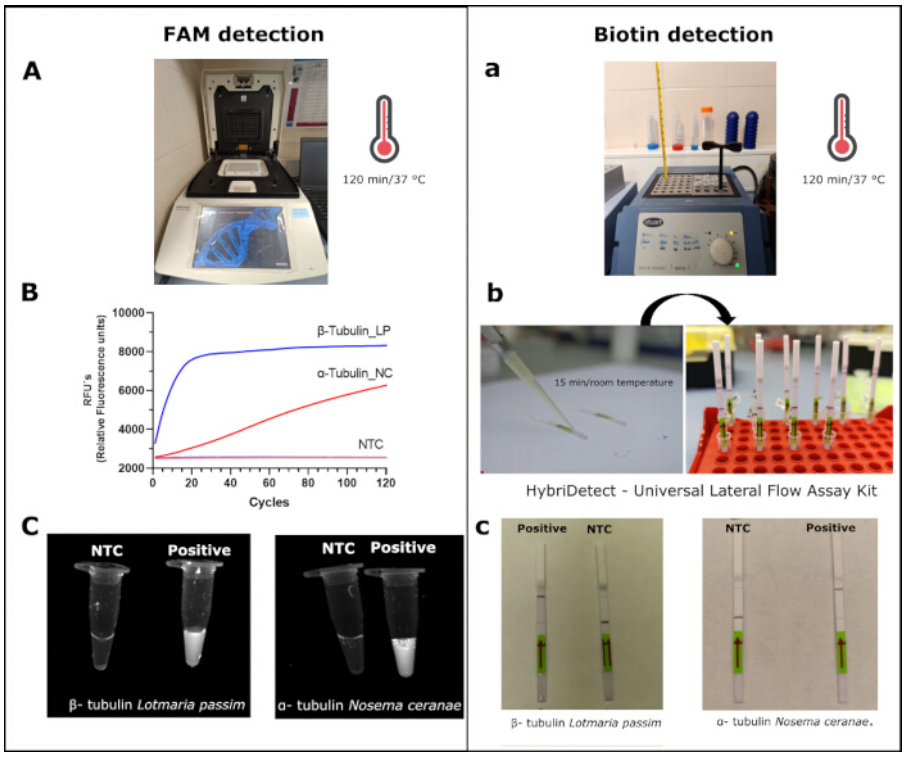

Figure 4: Detection of *Lotmaria passim* and *Nosema ceranae.* by the CRISPR/Cas12a method with FAM and biotin probes. For FAM detection, (**A**) samples are being incubated in a thermocycler; (**B**) the relative fluorescence units (RFU´s) of *L. passim* and *N.ceranae*; (**C**) visualization of the same samples under a photodocumentation system. In biotin tests, (**a**) a sample incubating in a thermo block; (**b**) samples were added in an immunostrip from the kit (see the **Table of Materials**); (**c**) the results after incubation. Abbreviations: LP= *Lotmaria passim*; NC= *Nosema ceranae*; NTC= no template control; RFU: relative fluorescence units Please click here to view a larger version of this figure.

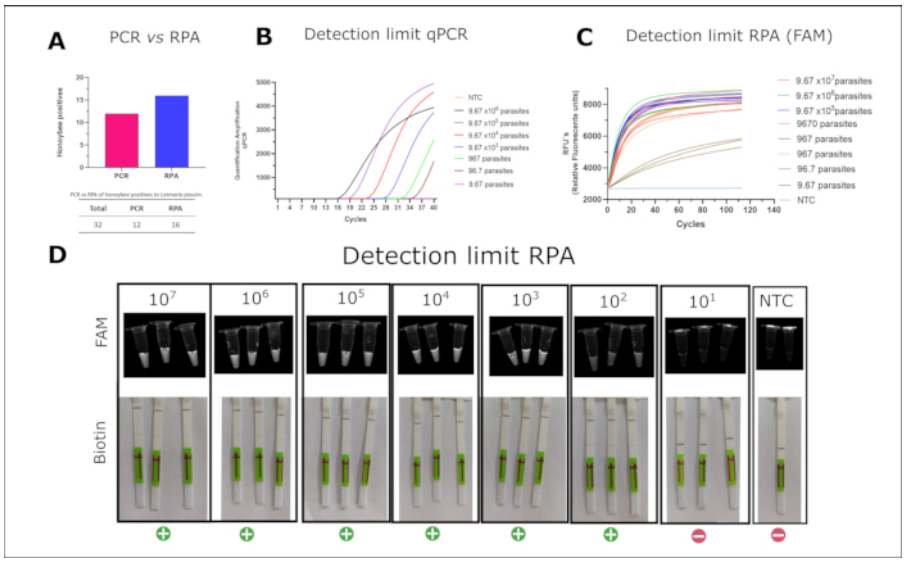

Figure 5: Comparison between qPCR and RPA, and detection limit of *Lotmaria passim*. (**A**) Detection of *L. passim* in honeybee samples using conventional qPCR and RPA. (**B**) qPCR detection limit of *L. passim* using SsoFast EvaGreen. (**C**) Detection limit of *L. passim* using CRISPR/Cas12a with a FAM-labeled probe; (**D**) visualization of the same CRISPR/Cas12a detection (from **C**) under a gel imaging system (top row), and corresponding lateral flow results using CRISPR/Cas12a with a biotin-labeled probe (bottom row). Abbreviations: RPA = recombinase polymerase amplification; qPCR= quantitative polymerase chain reaction. Please click here to view a larger version of this figure.

Reagent	Final Concentration	10 µL	50 µL
2x Reaction Buffer	1x	5 µL	25 µL
dNTPs (10 mM)	1.8 mM	1.8 µL	9 µL
10x Basic E-Mix	1x	1 µL	5 µL
Forward Primer (10 µM)	480 nM	0.5 µL	2.4 µL
Reverse Primer (10 µM)	480 nM	0.5 µL	2.4 µL
20x Core Reaction Mix	1x	0.5 µL	2.5 µL
DEPC Water	--	0.7 µL	3.7 µL

Table 1: Mastermix components for RPA with final volumes of 10 µL and 50 µL, and corresponding working concentrations:

Reagent	Final concentration	10 µL reaction	50 µL reaction
280 mM MgOAc	1.8 mM	0.5 µL	2.5 µL
DNA	10-0.1 ng	1 µL	2 µL

Table 2: Volume of reagents to be added per sample in a reaction of RPA

Reagent	[stock]	[final]	1x (µL)
Cas12a enzyme	1 µM	50 nM	2.5
cRNA	1 µM	100 nM	5
FAM probe	10 µM	250 nM	1.25
NEB buffer 10x	10x	1x	5
RPA amplicon	-	-	4
Water	-	-	32.25
Total			**50**

Table 3: Concentrations of reagents to make a mix of FAM detection by the method CRISPR/Cas 12a.

Reagent	[stock]	[final]	1x (µL)
Cas12a enzyme	1 µM	50 nM	2.5
cRNA	1 µM	100 nM	5
Biotin probe	10 µM	500 nM	2.5
NEB buffer 10x	10X	1X	5
RPA amplicon	-	-	4
Water	-	-	31
Total			**50**

Table 4: Concentrations of reagents to make a mix of biotin detection by the CRISPR/Cas 12a method.

Discussion

In this study, we developed and validated a practical molecular diagnostic protocol for detecting honeybee pathogens. The workflow integrates amplification-ready cell lysis, isothermal amplification using RPA, and highly specific detection via CRISPR/Cas12a. Among the chemical DNA

extraction methods evaluated, the HotSHOT alkaline lysis protocol offers significant advantages in cost, speed, and simplicity. It efficiently produces amplification-ready lysates without the need for commercial kits. This method has proven reliable in various applications, including pathogen detection in honeybees[14,15,16,17,18]. The high-temperature alkaline conditions disrupt cell membranes and hydrogen bonding between DNA strands, releasing single-stranded DNA suitable for downstream amplification. Although potential limitations include the presence of PCR inhibitors or suboptimal neutralization, alkaline lysis has been shown to work effectively in diverse organisms such as archaea[19], bacteria[20], and fungi[14], and is compatible with PCR, qPCR, and isothermal methods like LAMP[21] and RPA[12].

In our hands, the RPA/CRISPR/Cas12a system showed strong specificity and satisfactory sensitivity. The use of crRNAs targeting conserved regions in each pathogen allowed for accurate identification with no cross-reactivity to host DNA or unrelated microorganisms. Previous applications of this system in honeybees have achieved detection limits of approximately 6.5 × 10^2 and 6.2 × 10^1 copies/µL for Deformed Wing Virus types A and B[8,12]. However, these assays have not been optimized for in-field conditions with rapid RNA and/or DNA extraction. In this work, we adapted the method specifically for DNA pathogens -- *N. ceranae* and *L. passim* -- enabling field-compatible detection.

Our results show that RPA/CRISPR/Cas12a outperformed conventional PCR in identifying positive samples (16 vs. 12), likely due to the improved signal using the CRISPR/Cas12a collateral cleavage activity, which remains effective even in crude lysates. The improvement in sensitivity over PCR-based methods has been previously reported in different pathogen models such as *Yersinia enterocolitica*[22], *Diaporthe aspalathi*[23], or *Vibrio vulnificus*[24]. Furthermore, quantitative analysis revealed an equivalent limit of detection of RPA/CRISPR/Cas12a and qPCR under laboratory conditions (~96 parasite copies/µL). This implies a good performance of the method with comparable specificity to TaqMan qPCR, due to the added sequence recognition provided by the crRNA. Furthermore, the assay requires only minimal equipment, does not depend on a thermocycler or fluorescence reader, and can be visualized using lateral flow strips as previously developed for other pathogens[25,26]. This simplifies interpretation and makes the test highly accessible for field technicians and beekeepers.

The current protocol yields robust results, but further improvements are possible. Adapting the assay to target other DNA-based bee pathogens would only require redesigning RPA primers and crRNAs. For RNA viruses, the protocol can be expanded to include a reverse transcription step (RT-RPA) as previously demonstrated for several viral infectious diseases[26,27,28], including DWV-A and DWV-B in honeybees[15]. Additionally, the total assay time -- currently around 180 minutes -- can likely be reduced through protocol optimization for decreasing the timing of each step. Another alternative would be one-pot RPA/CRISPR/Cas12a that combines all RPA/CRISPR/Cas12a reagents in a single reaction. This all-in-one methodology has been successfully applied for the detection of a wide range of viral and bacterial pathogens[25,26,29]. Besides and despite that ssDNA probes have been extended and popularized for Cas12a detection, some studies have reported the capacity of Cas12a of trans-cleavage double-stranded DNA (dsDNA) probes that may be useful to be tested due to the stability of dsDNA[30]. This would lead to a time-to-result under 30 minutes, further enhancing field applicability. Lyophilizing RPA and CRISPR reagents would also improve reagent stability and eliminate

the need for a cold chain, increasing suitability for remote or low-resource environments. The successful performance of RPA/CRISPR/Cas12a using lyophilized reagents has been successfully determined for the detection of *Actinobacillus pleuropneumonia*[31] and respiratory infectious viruses[28,32], including SARS-Cov2[26].

In summary, this study presents a fully in-field, cost-effective, and portable molecular diagnostic platform for honeybee pathogen detection. This methodology, as it stands, would last 180 min approximately, but its total timing could still be subjected to further improvements. While we focused on *L. passim* and *Nosema* spp. as proof-of-concept targets, the system is modular and adaptable to a wide range of DNA-based bee pathogens (i.e., *Paenibacillus larvae* or *Melisococcus plutonious*) as well as for monitoring commensal symbionts of interest. This approach expands access to molecular diagnostics beyond traditional laboratory settings and supports early intervention strategies in apiculture. More broadly, our results demonstrate the viability of CRISPR-based technologies in field-ready diagnostic applications.

Disclosures

The authors have no conflicts of interest to disclose.

Acknowledgments

This work was supported by the grant "New molecular tools for rapid monitoring of honeybee diseases in apiaries in the province of Granada" (GOPG-GR-23-0002) under the call Aid to operational groups of the European Innovation Association (AEI) in agricultural productivity and sustainability of the Andalusian government. We also acknowledge the support by the Spanish Programme for Knowledge Generation and Scientific and Technological Strengthening of the R+D+I System, grant PID2021-126938OB-I00 funded by MCIN/AEI/10.13039/501100011033 and by "ERDF/EU". We strongly appreciated the help and resources provided by beekeepers of the Asociación de Pequeños Apicultores de Granada (APAG) and Cooperativas Agroalimentarias de Granada (FAECA). The study is part of the PhD thesis of D. Pedro García Olmedo. Doctoral Program in Fundamental and Systems Biology (University of Granada, UGR).

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