



# RPA/CRISPR-cas12a as a specific, sensitive and rapid method for diagnosing *Ehrlichia canis* and *Anaplasma platys* in dogs in Thailand

Suphaporn Paenkaew<sup>1</sup> · Nongluck Jaito<sup>2</sup> · Waranee Pradit<sup>3</sup> · Siriwadee Chomdej<sup>3</sup> · Korakot Nganvongpanit<sup>4,5</sup> · Puntita Siengdee<sup>6</sup> · Kittisak Buddhachat<sup>1,4</sup>

Received: 2 January 2023 / Accepted: 21 March 2023 / Published online: 30 March 2023  
© The Author(s), under exclusive licence to Springer Nature B.V. 2023

## Abstract

Rickettsial pathogens including *Ehrlichia canis* and *Anaplasma platys* are bacteria that cause parasitic infections in dogs such as canine monocytic ehrlichiosis (CME) and canine cyclic thrombocytopenia (CCT), respectively affecting mortality and morbidity worldwide. An accurate, sensitive, and rapid method to diagnose these agents is essential for effective treatment. In this study, a recombinase polymerase amplification (RPA) coupled with CRISPR-Cas12a methods was established to detect *E. canis* and *A. platys* infection in dogs based on the 16S rRNA. The optimal condition for DNA amplification by RPA was 37 °C for 20 min, followed by CRISPR-Cas12a digestion at 37 °C for one hour. A combination of RPA and the cas12a detection method did not react with other pathogens and demonstrated strong sensitivity, detecting as low as 100 copies of both *E. canis* and *A. platys*. This simultaneous detection method was significantly more sensitive than conventional PCR. The RPA-assisted cas12a assay provides specific, sensitive, rapid, simple and appropriate detection of rickettsial agents in canine blood at the point-of-care for diagnostics, disease prevention and surveillance.

**Keywords** *Ehrlichia* · *Anaplasma* · RPA · cas12a · Simultaneous detection

## Introduction

*Ehrlichia canis* and *Anaplasma platys* are obligate intracellular rickettsial pathogens that belong to the genera *Ehrlichia* or *Anaplasma* in the Anaplasmataceae family (Dumler et al. 2001). *Ehrlichia canis* and *A. platys* infect monocytes and platelets of dogs, respectively causing anemia and thrombocytopenia contributing to canine blood diseases such as canine monocytic ehrlichiosis (CME) and infectious canine cyclic thrombocytopenia (ICCT), respectively (Harrus and Waner 2011; Inokuma et al. 2002). Both these pathogens are transmitted by the brown dog tick (*Rhipicephalus sanguineus sensu lato*) through its saliva during the blood meal (Cicuttin et al. 2014; Fourie et al. 2013). *Ehrlichia canis* and *A. platys* are found in various regions around the world (Chomel 2011) including Asia (Pinyoowong et al. 2008), Europe (Cardoso et al. 2010), Africa (Nasr et al. 2020) and America (Almazán et al. 2016; Pérez-Macchi et al. 2019).

Microscopic examination of blood smears is the routine clinical diagnostic method to detect *E. canis* and *A. platys* morulae infections but these are difficult to identify in peripheral blood samples and occur in 4–6% of clinical

✉ Kittisak Buddhachat  
kittisakbu@nu.ac.th; k\_buddhachat@yahoo.com

<sup>1</sup> Department of Biology, Faculty of Science, Naresuan University, Phitsanulok 65000, Thailand  
<sup>2</sup> Enzyme Technology Research Team, Biorefinery and Bioproduct Technology Research Group, National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Paholyothin Rd., Klong Luang District, Pathum Thani 12120, Thailand  
<sup>3</sup> Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand  
<sup>4</sup> Excellence Center in Veterinary Bioscience, Chiang Mai University, Chiang Mai 50200, Thailand  
<sup>5</sup> Department of Veterinary Biosciences and Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50200, Thailand  
<sup>6</sup> Program in Applied Biological Sciences, Chulabhorn Graduate Institute, Chulabhorn Royal Academy, Kamphaeng Phet 6 Road, Laksi 10210, Bangkok, Thailand

cases (Mylonakis et al. 2003). Diagnosis of these agents by microscopic examination takes time and requires experienced veterinary practitioners (Gulati et al. 2013). Serological methods, such as the indirect immunofluorescence antibody (IFA) assay or enzyme-linked immunosorbent assay (ELISA) are useful diagnostic tools, which have been developed for point-of-care testing (POCT) (Waner et al. 2000). However, these methods are unable to differentiate species due to cross-reactions between species antibodies (Cárdenas et al. 2007). To combat these obstacles, molecular detection such as conventional PCR or real-time PCR have been employed to detect various pathogens with high specificity and sensitivity. These methods detect the pathogens' DNA, leading to reliable pathogen identity at species level (Buddhachat et al. 2020). These methods provide efficient detection but the requirement for high temperatures necessitates the use of a thermal cycler, which is rarely available at POCT (Kramer and Coen 2001).

Isothermal nucleic acid amplification methods are widely used to accelerate diagnostic applications such as loop-mediated isothermal amplification (LAMP) or recombinase polymerase amplification (RPA) (Fu et al. 2011; Rohrman et al. 2012). These methods enable amplification of DNA or RNA at constant temperature with minimal instrumentation and energy such as a heat block, water bath or even body temperature to perform the reaction, contributing to feasible adaptation for POCT. RPA can also amplify DNA or RNA through a variety of enzymatic processes, including the disassociation and replication of double-stranded DNA. The RPA reaction can be carried out at temperatures ranging from 25 °C to 45 °C, with optimal temperature 37 °C and amplification duration 5–20 min (Li and Macdonald 2015). CRISPR-Cas systems have frequently been used in combination with DNA amplification for pathogen detection, providing signal amplification and simple assay readout (Chen et al. 2018; Gootenberg et al. 2018). Cas proteins including cas12a, cas12b and cas13 show enhanced specificity and sensitivity for pathogen detection, with inherent collateral activity to cleave single-stranded DNA or RNA as the reporter molecules to show the presence or absence of the DNA portion of interest (like pathogens and SNP) (Li et al. 2019; Lu et al. 2020; Qin et al. 2019). These proteins are simple to detect and minimal instrumentation is required such as a heat block, water bath or body temperature (Aman et al. 2020). Combination of RPA and the cas12a assay is an appropriate pathogen diagnostic in POCT (Wang et al. 2019). RPA-assisted cas12a has been applied in shrimp pathogen diagnosis to detect WSSV (white spot syndrome virus) (Chaijarasphong et al. 2019) and also used for the detection of plant viruses (Aman et al. 2020) and bacteria (Buddhachat et al. 2022).

This study combined the RPA and cas12a assays as a novel method for detecting *E. canis* and *A. platys* rickettsial

infection in dogs from peripheral blood. Species-specific primers for DNA amplification and gRNA target were designed based on the 16S rRNA region. We also determined the specificity and sensitivity of the assay for *E. canis* and *A. platys* detection. Clinical blood samples were used to validate the use of the established approach.

## Materials and methods

### DNA samples and DNA plasmid preparation

Synthetic plasmids pUC57 harboring partial 16S rRNA of *E. canis* (accession number KY594915) and *A. platys* (accession number KJ659044) as well as pUC57 harboring partial 18S rRNA of *Babesia vogeli* (accession number MH143394) and *Hepatozoon canis* (accession number MK091088) were synthesized from MacroGen (Korea). The synthetic plasmids were transformed into heat-shocking competent *E. coli* DH5 alpha cells (Invitrogen, USA). Bacterial cells containing the synthetic plasmids were cultured in LB broth with 100 µg/ml ampicillin to produce plasmids as the positive control. The plasmids were isolated using NucleoSpin Plasmid EasyPure kit (Macherey–Nagel, Germany) and the quality and quantity of plasmids obtained were measured using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, USA). DNA materials of different bacterial pathogens including closely-related species: *Wolbachia* sp., a bacterial pathogen in the blood of dogs or cats: *Leptospira* sp. (Ls) and *Mycoplasma* sp. (Ms) were gifted from the Animal Molecular Diagnostic Service (AMDS) (Chiang Mai, Thailand), while other bacterial species: *Escherichia coli* (Ec), *Bacillus cereus* (Bc), *Staphylococcus epidermidis* (Se), *Staphylococcus aureus* (Sa), *Klebsiella oxytoca* (Ko) and *Citrobacter freundii* (Cf) were available in our laboratory. Other protozoa enabling infection in the blood of dogs, cats or other mammals consisting of *Babesia vogeli* (Bv), *Babesia gibsoni* (Bg), *Babesia bovis* (Bb), *Hepatozoon canis* (H), *Trypanosoma evansi* (Te) and *Theileria* sp. (Ts) were gifted from AMDS. These pathogens were used for analytical specificity determination of the assay.

### RPA primer design

RPA primers used to amplify the DNA of *E. canis* and *A. platys* were modified from Bact16S-A primers of our previous study (Buddhachat et al. 2020) by increasing the 5' end extension of primer length up to 33 bp. The primer pair was designed from partial sequences of 16S rRNA from different species by multiple alignments using MultAlin (<http://multalin.toulouse.inra.fr/multalin/>). The 16S rRNA was employed for two reasons. Firstly, we utilized the available 16S rRNA sequences of various rickettsia species in

GenBank. Secondly, based on our previous study, the 16S rRNA contained sufficient variation in nucleotides between *E. canis* and *A. platys* to differentiate by high resolution melting analysis (Buddhachat et al. 2020). Accession numbers of sequences used in this study were retrieved from the National Center for Biotechnology (NCBI; <https://www.ncbi.nlm.nih.gov/>). Features of the designed primers were determined using an OligoAnalyzer (<https://sg.idtdna.com/pages/tools/oligoanalyzer>). The designed RPA primers are listed in Table 1 and Supplemental Figure S1.

**RPA reaction**

The RPA reaction was performed for DNA amplification based synthetic DNA plasmid containing partial 16S RNA region of *E. canis* and *A. platys* using a TwistAmp® Basic kit (TwistDx, England). A 25 µl volume of RPA reagent consisted of 1X reaction buffer, 1X Basic E-mix, 1.8 mM dNTPs, 0.48 µM of each primer of EA\_16S, 0.48 µM of each primer of BH\_18S (Table 1), 1X core reaction mix, 20 ng of DNA template of either *E. canis* and *A. platys* and 14 nM magnesium acetate and then nuclease-free water was added up to 25 µl. For negative control, nuclease-free water was added instead of DNA template. The reagent was placed in a PCR tube and the optimal temperature was determined by varying the temperature from 25, 30, 35, 37, 40 and 45 °C for 20 min. The RPA products obtained were subjected to 1.5% ethidium bromide (EtBr)-stained agarose gel electrophoresis to visualize the amplicon under a UV transilluminator. A suitable temperature obtained from this experiment was used for DNA amplification by RPA in the next independent experiments.

**Design and synthesis of gRNA**

The guide RNA (gRNA) for the cas12a assay was designed based on multiple alignments of 16S rRNA sequences of *E. canis* and *A. platys* amplified from the RPA primer under MultAlin (<http://multalin.toulouse.inra.fr/multalin/>). Criteria

of the gRNA design included (i) looking for protospacer adjacent motif (PAM) nucleotide sequence 5'-TTTV-3' (where V is A, C, or G) as the specific region for only cas12a to unwind the DNA target and (ii) selecting one DNA region specific to *E. canis* or another specific to *A. platys* at next to the 3' end of the PAM site 18–23 bp in length, with sequences after the PAM site used for the spacer of gRNA. To attain more specificity for the cas12a assay, each of the species-specific gRNA sequences was suggested as the base difference within 3–6 bp next to the PAM position (Zetsche et al. 2015). The gRNA was synthesized using in vitro transcription by HiScribe™ T7 High Yield RNA Synthesis kit (E2050S, NEB, England). The synthetic double-stranded DNA as the template to generate gRNAs was purchased from Integrated DNA Technologies (IDT, USA). We designed dsDNA comprising the T7 promotor, tracrRNA for cas12a recognition and spacer sequences or crRNA to specifically bind to DNA targets of either *E. canis* or *A. platys* (Table 1 and Supplemental Figure S2). After RNA transcription, the Monarch RNA cleanup kit was used to purify the synthesized gRNA and eliminate contamination (NEB, England). Concentration of the synthesized gRNA was measured using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis. gRNA concentration was adjusted to 10 µM for in vitro digestion of the cas12a assay.

**In vitro digestion of the cas12a assay**

A constant 1:1 ratio was used to form the binary complex of cas12a and gRNA, while the final concentration of cas12a:gRNA was varied from 12.5:12.5 nM to 150:150 nM to find the suitable concentration to visualize the fluorescence signal from the cas12a assay. A cas12a or cpf1 (#M0653T, NEB, US) cleavage reaction of a total volume of 30 µl comprised 1X 2.1 NEB buffer, 100 nM cas12a, 100 nM gRNA\_E or gRNA\_A, 1 µl of 1.6 µM single-stranded DNA reporter (ssDNA reporter) (Integrated DNA Technologies, USA), 5 µl of the RPA product obtained from all reactions and added nuclease-free water to 30 µl. The

**Table 1** Primer and probe sequences used in the experiment

Method	Primer name	Sequence (5'-3')	Species detection	Reference
RPA-assisted cas12a	EA_16S	F: CAAGCGTTGTTTCGGAATTATTGGG CGTAAAGGG R: ACGAATTTACCTCTACACTAGGAATTCGCTA	<i>E. canis</i> <i>A. platys</i>	Modified from Buddhachat et al. 2020
	BH_18S	F: CTGGTAATTCCGTTAACGAACGAGACCTTAA R: GGGCATCACAGACCTGTTATTGCCTYAAACTTC	<i>Babesia</i> spp. <i>H. canis</i>	
	gRNA_E	TTTGGAGCGGCTTTTAATACTGC	<i>E. canis</i>	In this study
	gRNA_A	TTTCACCTTTAACTTACCGAACCGCC	<i>A. platys</i>	In this study
	ssDNA reporter	56-FAM TTATTATT 3IABkFQ		
Real-time PCR	Bact16S-A	F: GTTCGGAATTATTGGGCGTAAAGGG R: CACCTCTACACTAGGAATTCGCTA	<i>E. canis</i> <i>A. platys</i>	Buddhachat et al. 2020

binary complex of cas12a and gRNA was formed before mixing the other chemicals at under 37 °C for 10 min. The cas12a reaction was performed at 37 °C for one hour. The fluorescence signal was recorded using a real-time PCR machine for data acquisition during one hour of the assay. The end-point detection of fluorescence was observed under an LED transilluminator.

### Specificity determination of the RPA-assisted cas12a assay

To determine whether an RPA-assisted cas12a assay using EA\_16S could specifically detect *E. canis* and *A. platys* without cross-reaction with other pathogens, DNA samples of other infectious agents including *E. canis* (E), *A. platys* (A), *Wolbachia* sp. (Ws), *Leptospira* sp. (Ls), *Mycoplasma* sp. (Ms), *Escherichia coli* (Ec), *Bacillus cereus* (Bc), *Staphylococcus epidermidis* (Se), *Staphylococcus aureus* (Sa), *Klebsiella oxytoca* (Ko), *Citrobacter freundii* (Cf), *Babesia vogeli* (Bv), *Babesia gibsoni* (Bg), *Babesia bovis* (Bb), *Hepatozoon canis* (H), *Trypanosoma evansi* (Te) and *Theileria* sp. (Ts) were used as DNA templates at 20 ng/μl. RPA products obtained were detected using 1.5% agarose gel electrophoresis with EtBr staining and visualized under a UV transilluminator. Subsequently, the RPA products were detected for the presence of DNA target by in vitro digestion of the cas12a assay using either gRNA\_E for *E. canis* or gRNA\_A for *A. platys* to determine the specificity of gRNA\_E and gRNA\_A. The fluorescence signal from ssDNA degradation by cas12a was recorded by real-time PCR. DNA plasmids harboring either partial 16S rRNA or 18S rRNA of four pathogens including *E. canis* (E), *A. platys* (A), *B. vogeli* (B) and *H. canis* (H) often detected in canine blood in both single or coinfection in Thailand (Yabsley et al. 2008) were combined as EA, EB, EH, AB, AH, BH, EAB, EAH, ABH, EBH and EABH for species authentication in mimicking coinfection by the RPA-assisted cas12a assay, and their fluorescence signals were observed every hour for four hours.

### Sensitivity determination of the RPA-assisted cas12a assay

DNA plasmids harboring partial 16S rRNA of *E. canis* and *A. platys* were diluted at 10<sup>8</sup> to 1 copies/μl to evaluate the sensitivity of the RPA-assisted cas12a assay. RPA reactions were performed using EA\_16S primer with DNA dilutions of either *E. canis* and *A. platys*. The RPA products obtained were then assayed by in vitro cas12a digestion under a heat block at 37 °C, and the fluorescence signal was recorded by a real-time PCR (Bioer, China) every minute for one hour. Based on the acquired fluorescent signal from the cas12a assay using gRNA\_E and gRNA\_A, the limit of detection

(LoD) was determined using the formula  $LoD = LoB + 1.645 (SD_{low\ concentration\ sample})$  (Armbruster and Pry 2008) where the limit of blank (LoB) is the mean of the blank sample and test replicate of the blank as per the following formula:  $LoB = mean_{blank} + 1.645 (SD_{blank})$  (Details of LoD determination were explained in Supplemental File 1). At assay completion, the reaction tube was inspected under an LED transilluminator to view the fluorescence signal with the naked eye.

### RPA-assisted cas12a assay for hemopathogen detection in clinical blood samples

Thirty DNA samples isolated from the blood of dogs with clinical signs for possible hemopathogen infection and five DNA samples of healthy dogs were provided from the Animal Molecular Diagnostic Service (AMDS), Chiang Mai, Thailand to assess the performance of the RPA-assisted cas12a assay for *E. canis* and *A. platys* detection in real clinical samples and determine the presence of both agents. To determine whether a tested sample was positive or negative, the fluorescence signal of the negative control was used as the threshold. If a sample gave a fluorescence signal higher than the negative control, it was considered as positive. If the opposite, it was considered as negative. The high resolution melting (HRM) assay using Bact16S-A primers (Buddhachat et al. 2020) was employed to detect *E. canis* and *A. platys* along with sequencing some PCR products to confirm the results among different approaches. An overview of the rickettsial detection process by the RPA-assisted cas12a assay is illustrated in Supplemental Figure S3.

### Statistical analysis

The fluorescence signal obtained from the specificity of the RPA-assisted cas12a assay using either gRNA\_E or gRNA\_A was compared between the fluorescence signal of *E. canis* or *A. platys* and the others using the Student's t-test with significant difference set at  $p < 0.05$ . A confusion matrix was also created to determine the performance of the RPA-assisted cas12a assay compared to the HRM. Accuracy and precision of the HRM and the RPA-assisted cas12a assays were calculated as follows:

$$\text{Accuracy} = \frac{\text{true positive} + \text{true negative}}{\text{total samples}}$$

$$\text{Precision} = \frac{\text{true positive}}{\text{true positive} + \text{true negative}}$$

where true positive is a sample with a positive result for both RPA-assisted cas12a assay and HRM and true negative is a

sample with a negative result for both RPA-assisted cas12a assay and HRM.

We used Cohen's kappa to analyze the correlation coefficient as the degree of agreement between the two methods as follows:

$$\text{kappa (K)} = \frac{\text{actual observed positive and negative agreement} - \text{chance agreement}}{1 - \text{chance agreement}}$$

## Results

### RPA-assisted cas12a assay optimization

RPA primers modified based on the conserved 16S rRNA region of *E. canis* and *A. platys* showed amplicon size in agreement with the expected size of 145 bp under the RPA condition incubated at 25 °C to 40 °C for 20 min (Supplemental Figure S1B). We selected 37 °C for DNA amplification as the optimal RPA reaction temperature for further study.

We designed two species-specific gRNAs for either *E. canis* or *A. platys* from the sequence within amplicons, given as gRNA\_E and gRNA\_A (Supplemental Figure S2C and Table 1). The gRNAs were synthesized by in vitro transcription using T7 RNA polymerase, as depicted in Supplemental Figure S3C. For the in vitro cas12a digestion, concentrations of cas12a: gRNA at a ratio of 1:1 were varied. The fluorescence signal from cleavage of the ssDNA reporter was clearly observed by the naked eye at 100 nm for both cas12a and gRNA after incubation (Supplemental Figure S3D). An increase in the fluorescence signal when extending incubation time for 2 h was also observed (Supplemental Figure S4).

### Specificity of the cas12a assay

After the RPA and in vitro digestion of cas12a were established, the two processes were combined to detect both *E. canis* and *A. platys*. The specificity of the RPA-assisted cas12a assay was determined by comparing the DNA of different species. The RPA used EA\_16S primers to amplify plasmid DNA harboring the 16S rRNA of *E. canis* and *A. platys* with a 145 bp-amplicon but no DNA amplification for both bacterial and protozoan agents (Fig. 1A). Subsequently, RPA reagents from eight species were used as targets for in vitro cas12a digestion using either gRNA\_E for *E. canis* or gRNA\_A for *A. platys*. A positive fluorescence signal for the cas12a assay using gRNA\_E was noted in only *E. canis*. Similarly, *A. platys* gave a positive fluorescence signal for the cas12a assay using gRNA\_A. The DNA of other pathogens showed no fluorescence signal from cleavage of the ssDNA reporter

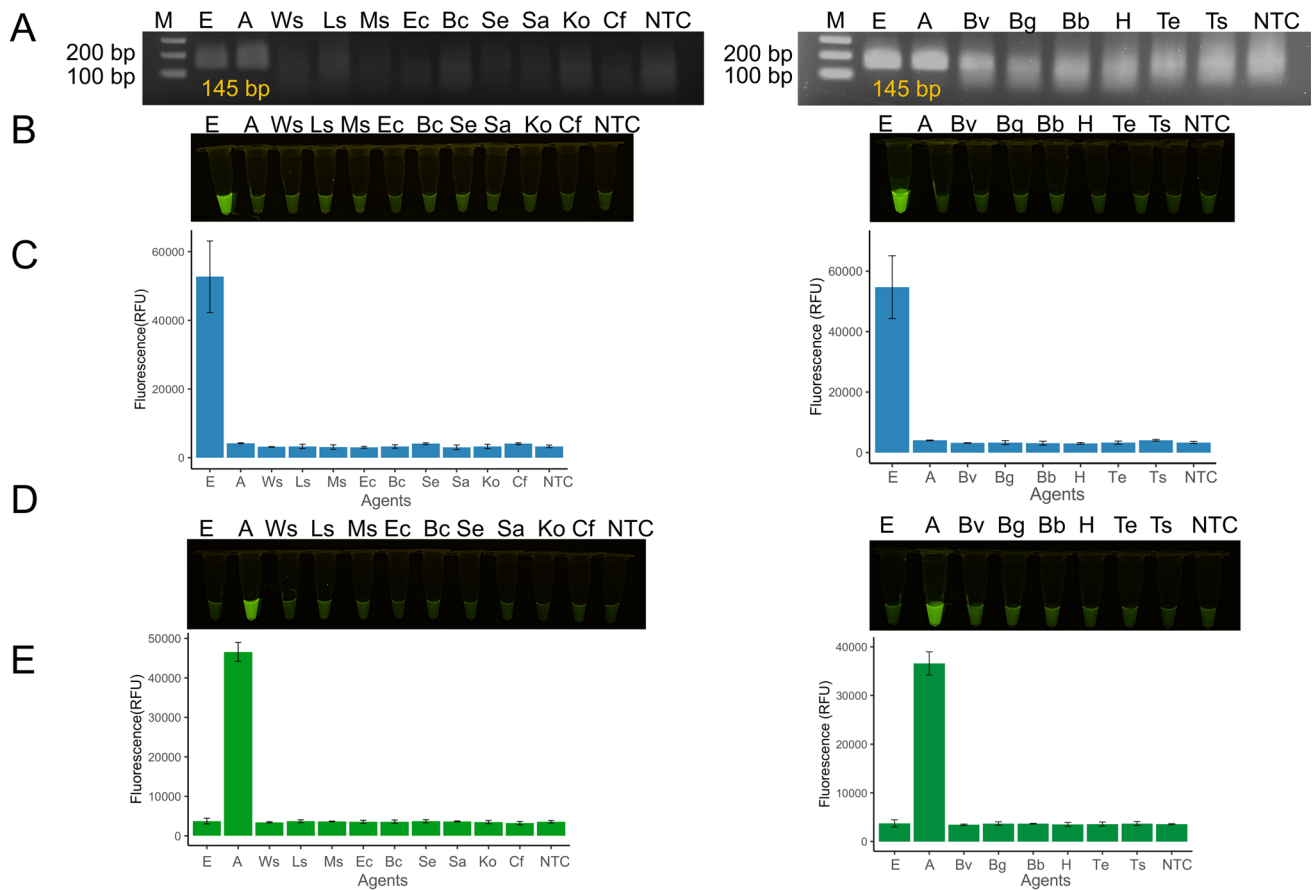
(LoD) from the collected fluorescence signal values at approximately 163 and 125 copies for detecting *E. canis* and *A. platys*, respectively by this method (Fig. 3E and F). The limit of detection determination is shown in Supplemental Figure S6 and Supplementary file 1.

(Fig. 1B and D). The value of the fluorescence signal measured by real-time PCR and the fluorescence signal for *E. canis* measured by the cas12a assay using gRNA\_E, and for *A. platys* using gRNA\_A showed considerably significant differences from the others ( $p < 0.05$ ) (Fig. 1C and E).

We also tested the specificity of the RPA-assisted cas12a assay in the admixture of DNA from distinct species in one sample as mimicking coinfection with two, three, or four canine species. Agarose gel electrophoresis results indicated that all combinations of admixtures (two, three, and four species) showed positive RPA products (Fig. 2A). When these RPA products were detected by the cas12a assay using specific gRNA including either gRNA\_E or gRNA\_A, the fluorescence signal appeared in some tubes that contained species DNA targets. For the cas12a assay using gRNA\_E, the admixture including *E. canis* amplicon gave a fluorescence signal, and in the same way for the cas12a assay using gRNA\_A, a positive fluorescence signal was observed in the tube with *A. platys* amplicon (Fig. 2B and C). We noticed that the cas12a assay using gRNA\_E incubated at 37 °C for four hours enabled cross-activity with the other species (Supplemental Figure S5A). Longer incubation of the cas12a assay using gRNA\_A showed no cross-reaction with the other species (Supplemental Figure S5B).

### Sensitivity of the RPA-assisted cas12a assay detection

To determine the sensitivity of the RPA-assisted cas12a assay, the synthetic plasmid DNA samples harboring 16S rRNA of *E. canis* and *A. platys* were diluted at  $10^8$  to 1 copy numbers and used as DNA templates for amplification by the RPA reaction. Results showed that the amount of DNA noticeable on agarose gel electrophoresis was as little as  $10^5$  and  $10^4$  copies of *E. canis* and *A. platys*, respectively (Fig. 3A and B). When the RPA products obtained were detected by the cas12a assay using either gRNA\_E or gRNA\_A, a fluorescence signal was noted at the least concentration of  $10^2$  copies for both, as shown in Fig. 3C and D. We also calculated the limit of detection



**Fig. 1** RPA-assisted cas12a assay specificity for single infection of either *E. canis* or *A. platys*. (A) agarose gel electrophoresis of the RPA reaction using EA\_16S primer for DNA amplification with DNA samples of other infectious agents including *E. canis* (E), *A. platys* (A), *Wolbachia* sp. (Ws), *Leptospira* sp. (Ls), *Mycoplasma* sp. (Ms), *Escherichia coli* (Ec), *Bacillus cereus* (Bc), *Staphylococcus epidermidis* (Se), *Staphylococcus aureus* (Sa), *Klebsiella oxytoca* (Ko), *Citrobacter freundii* (Cf), *Babesia vogeli* (Bv), *Babesia gibsoni* (Bg), *Babesia bovis* (Bb), *Hepatozoon canis* (H), *Trypanosoma*

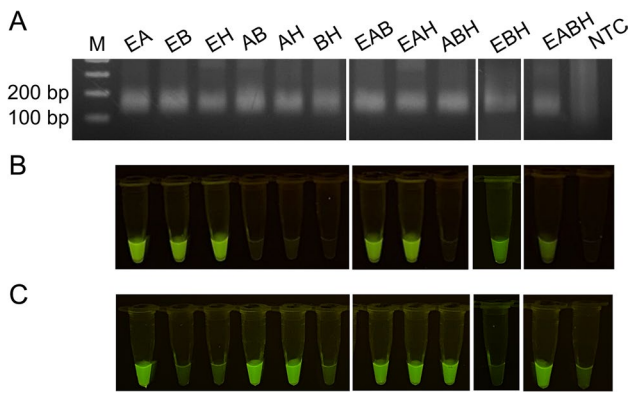
*evansi* (Te) and *Theileria* sp. (Ts). (B) cas12a assay using gRNA\_E to detect the RPA products of each species using LED light under an LED transilluminator, allowing examination by the naked eye. (C) Fluorescence signal from cleavage of the ssDNA reporter by the cas12a assay using gRNA\_E. (D) cas12a assay using gRNA\_A to detect the RPA products of each species. Detection using an LED transilluminator allowed examination by the naked eye. (E) Fluorescence signal of the cas12a assay using gRNA\_A collected by real-time PCR at 37 °C for one hour. NTC represents non-template control

### RPA-assisted cas12a assay for detecting rickettsial agents in clinical samples

Thirty-five DNA samples isolated from real canine blood were used to amplify DNA by the RPA reaction, followed by the cas12a assay to diagnose *E. canis* or *A. platys*. Examination of pathogen infection determined whether it was positive or negative based on the fluorescence signal value of the negative control (Supplemental Figure S7A and B). Results showed 28 and 23 positive samples for *E. canis* and *A. platys*, respectively with 20 samples showing coinfection for both *E. canis* and *A. platys* (Fig. 4B and C).

We compared HRM with the RPA-assisted cas12a assay for the detection of *E. canis* and *A. platys*. Almost

all positives from the RPA-assisted cas12a assay gave positive results by HRM. Five and six samples of *E. canis* and *A. platys*, respectively were positive for the RPA-assisted cas12a assay but negative for HRM (Fig. 4A and Supplemental File 2). Some positive samples with HRM were also sequenced for species confirmation with six and one samples out of 18 samples exhibited sequences similar to *E. canis* and *A. platys*, respectively. To evaluate the performance of the RPA-assisted cas12a assay against HRM, a confusion matrix was performed to derive the precision and accuracy of the two approaches (Table 2). Precision and accuracy obtained from the RPA-assisted cas12a assay compared to HRM were 82% and 86% for *E. canis* detection and 78% and 86% for *A. platys* detection. The kappa statistic was 0.65 for *E. canis* and 0.66 for *A. platys* detections with a



**Fig. 2** RPA-assisted cas12a assay specificity of the admixture of various pathogens including *E. canis*, *A. platys*, *B. vogeli* and *H. canis* under 37 °C for one hour. (A) Agarose gel electrophoresis of the RPA reaction for combined DNA amplification including *E. canis* (labeled E), *A. platys* (labeled A), *B. vogeli* (labeled B) and *H. canis* (labeled H) using BH\_18S and EA\_16S primers. The cas12a assay was performed to detect the RPA products of combined DNA using gRNA\_E (B) and gRNA\_A (C). PCR tubes were examined under an LED transilluminator by the naked eye. NTC represents non-template control

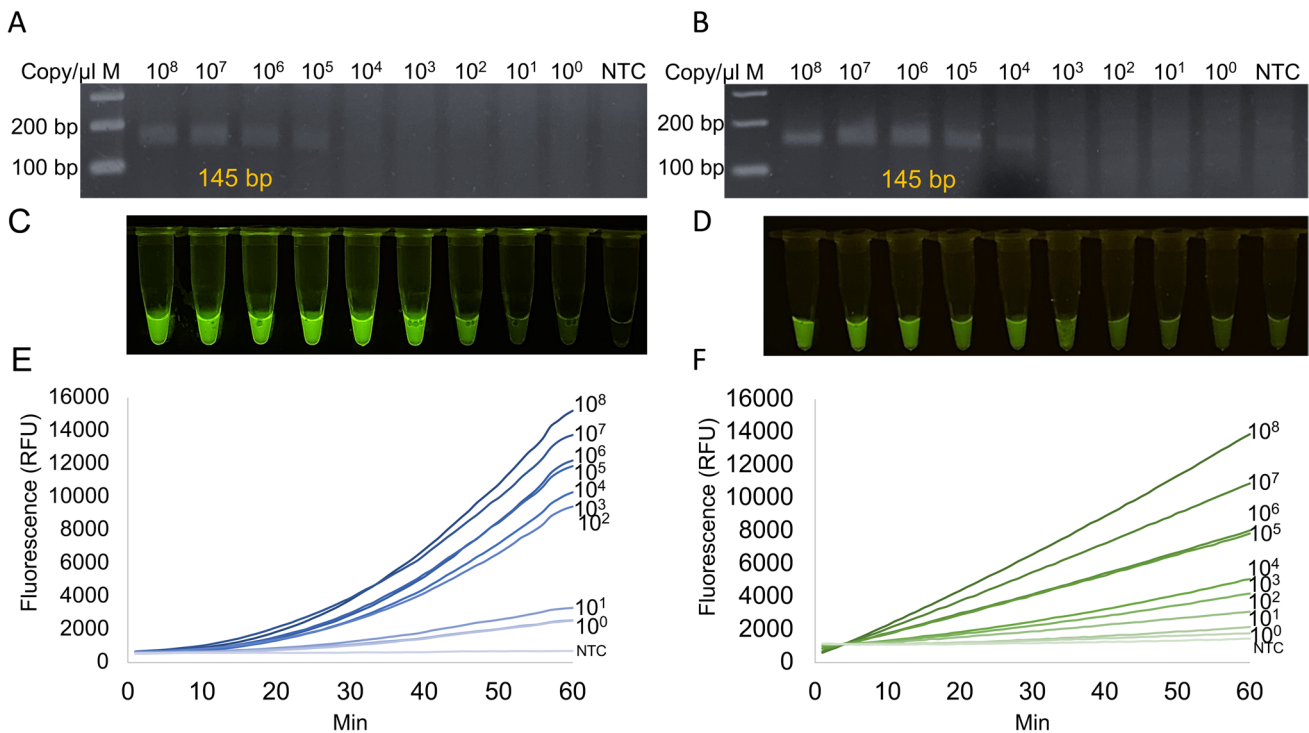
substantial level of agreement. The sequence results showed low success rate and one sample appeared to be positive for *A. platys* in contrast to the sequence result exhibiting *E.*

*canis*. The sequencing result of species prediction is shown in Supplemental File 3.

### Discussion

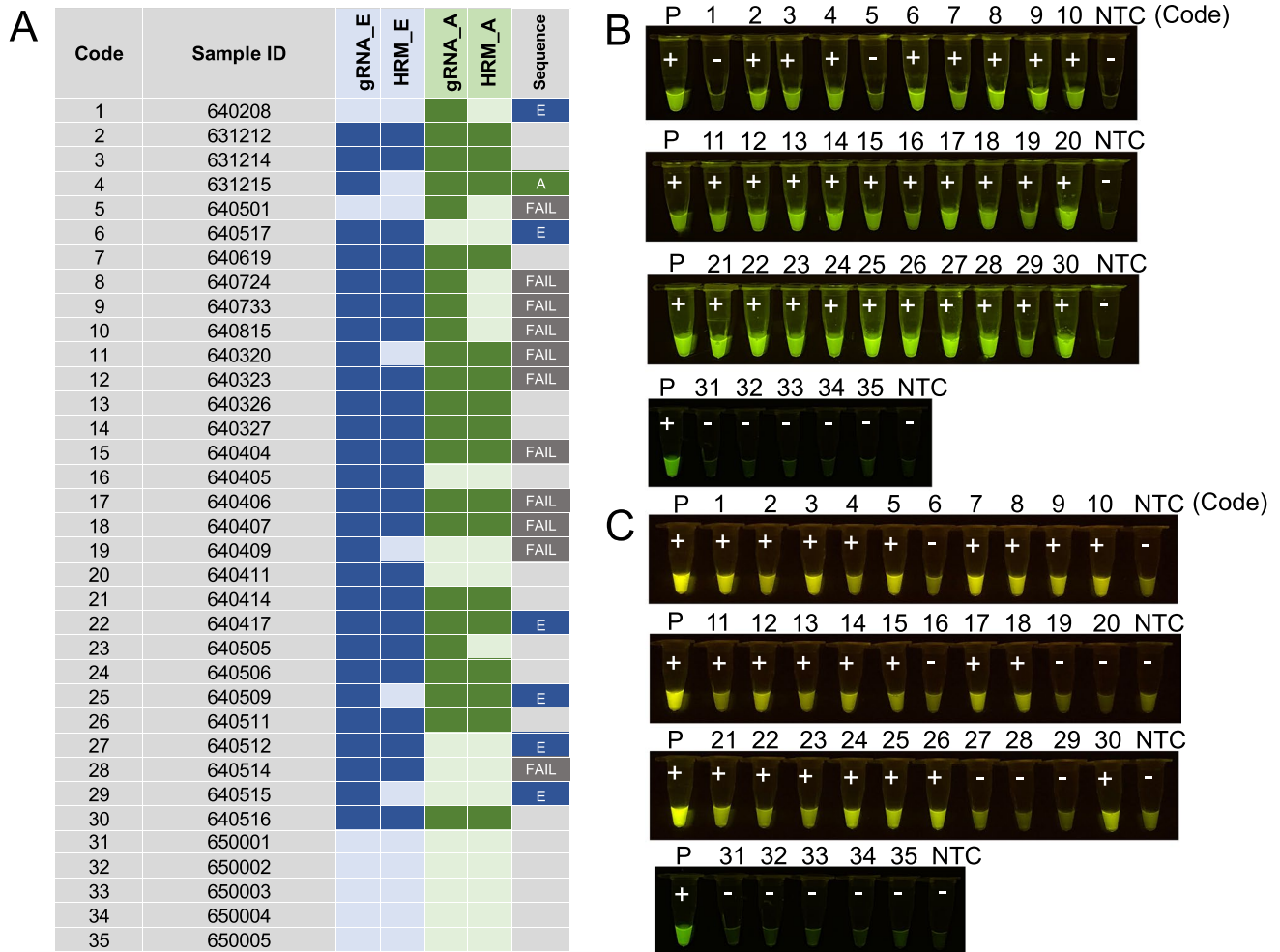
The CRISPR-Cas system has been extensively deployed to accelerate the highly efficient diagnostic toolkit with speed, specificity and sensitivity, relying on gRNA binding with target sequences on the DNA or RNA target site (Kim et al. 2021). Therefore, extensive cas proteins such as cas12 and cas13 were coupled with isothermal nucleic acid amplification for pathogen detection; for example, cas13 for SARS-CoV-2 and RAA-cas12a for African swine fever virus detection (Chandrasekaran et al. 2022; Wang et al. 2020). Our findings revealed that RPA for amplification and cas12a for detecting the DNA target was successfully harnessed to diagnose *E. canis* and *A. platys* with specificity, sensitivity and simplicity that required minimal instruments, and was suitable for POCT.

In this study, the suitable concentration and ratio of cas12a and gRNA to form a binary complex was cas12a:gRNA 100:100 nM. Previous studies demonstrated different results. For instance, the optimal condition of



**Fig. 3** Sensitivity of the RPA-assisted cas12a assay. Agarose gel electrophoresis of ten-fold dilutions of the DNA plasmid used in the RPA amplification DNA of *E. canis* (A) and *A. platys* (B). The RPA-assisted cas12a assay using gRNA\_E (C) and gRNA\_A (D) was conducted under an LED transilluminator, with measurement of

the fluorescence signal from cleavage of the ssDNA reporter by real-time PCR at 37 °C for one hour of *E. canis* (E) and *A. platys* (F) for DNA plasmid dilution. NTC represents non-template control. Limit of detection was calculated using the fluorescence signal value of ssDNA cleavage with three replicates



**Fig. 4** RPA-assisted cas12a assay compared with HRM results. RPA-assisted cas12a assay using gRNA\_E and gRNA\_A compared with HRM to detect *E. canis* and *A. platys* (A). The tubes were placed under an LED transilluminator for RPA-assisted cas12a detection

**Table 2** Precision and accuracy of the RPA-assisted cas12a assay compared with HRM detection

Method	Precision (%)	Accuracy (%)	Cohen’s kappa
<i>E. canis</i> detection	82	86	0.65
<i>A. platys</i> detection	78	86	0.66

the cas12a platform for mycoplasma contamination detection was concentration of gRNA:cas12a as 500:250 nM at 37 °C for 30 min or hepatitis C virus detection as 40 nM of cas12a:gRNA with detection within 30 min. These studies applied isothermal amplification including RPA or LAMP coupled with cas12a that demonstrated 100% accuracy for detection (Kham-Kjing et al. 2022). We illustrated the effectiveness of the RPA-assisted cas12a assay for identifying rickettsial agents. This approach involves a few steps,

using gRNA\_E (B) and gRNA\_A (C). Blue is positive result of *E. canis*, green is positive result of *A. platys*. NTC represents non-template control. HRM results are shown in Supplemental File 1

including DNA amplification using RPA, which takes only 20 min. Subsequently, the cas12a assay was utilized for detecting the rickettsial DNA, which requires an additional hour. The entire process without DNA extraction can be completed in just 90 min under simple heat block at a temperature of 37 °C. However, temperature range 25 to 40 °C enabled amplification of the DNA target by RPA. We selected the tested temperature at 37 °C because this was the optimal temperature of subsequent in vitro digestion of cas12a and we utilized body temperature to assess this approach in a limited resource area (Crannell et al. 2014).

The RPA-assisted cas12a assay displayed explicit specificity for DNA bacteria of *E. canis* and *A. platys* detection without cross-reaction to other pathogens under the optimal condition, indicating that cas12a detection showed endurance with mismatch sequences or no targets from RPA amplification. The primers for the RPA process selected a



similar region of 16S rRNA between *E. canis* and *A. platys* but distinguished from other hemopathogens infected in dogs or other mammals. The primer amplified *E. canis* and *A. platys* 16S rDNA, resulting in an increased amount of DNA target, with gRNA then designed from the region with species-specific sequence on the DNA target amplified. The specific gRNAs of *E. canis* and *A. platys* have single nucleotide variation at the third and fifth position from the 3' end next to PAM. The seed region of gRNA which is mismatch intolerant is at the first five nt next to PAM (Zetsche et al. 2015). Different nucleotides, especially single nucleotide polymorphism (SNP) on the seed region and the PAM site, caused both gRNAs to have specific detection based on similar amplification regions. The specificity determination showed that gRNAs could only bind with DNA targets because the fluorescence signal from the collateral activity is significantly different between DNA targets and other DNA pathogens. Our previous study demonstrated that RPA combined with cas12a showed promise for species differentiation of *Phyllanthus amarus* from other closely related species, based on the difference in PAM sequence and 4–5 nucleotides at the 5' end (seed sequence) of the spacer. These features can be used to facilitate target-specific detection in the DNA region of interest of certain organisms (Buddhachat et al. 2021; Gerashchenkov et al. 2020). We also evaluated the specificity of the RPA-assisted cas12a assay using either gRNA\_E or gRNA\_A in complex condition, with admixture of DNA templates of various hemopathogens like coinfection. Our findings exhibited that although the DNA templates were admixed with the DNA of other pathogens, the assay using either gRNA\_E or gRNA\_A also gave the fluorescence signal in only the condition with the presence of *E. canis* or *A. platys*, respectively. This suggested that the DNA of the other pathogens did not interfere with the assay.

However, we noted that the RPA-assisted cas12a assay using gRNA\_E was incubated for longer than two hours, enabling nonspecific detection with *A. platys* DNA but not for the RPA-assisted cas12a using gRNA\_A. Therefore, the gRNA\_E showed binding DNA of *A. platys* in a similar region because the sequence of gRNA\_E was different from the DNA of *A. platys* by only one base pair from the SNP of the seed region. The gRNA\_E may have cross-activity with other species because the DNA sequences of different agents are similar (Unver et al. 2003). Moreover, incubating reactions for a long time resulted in cas-gRNA complex binding and cutting of non-specific targets that presented cleavage fluorescence signals of no targets. Consequently, the study for cas12a detection used the optimal concentration of cas12a:gRNA and the appropriate time for detection (Buddhachat et al. 2022, 2021; Ding et al. 2020; Mukama et al. 2020). However, the cas12a assay specifically detected different DNA within one hour, thereby saving time. The cas12a-gRNA complex distinguished types

of pathogens in one reaction; therefore, proposing a cas12a detection system for multiplex pathogen detection in a one-pot reaction. Previously, Cas13 was developed to detect multiplex Dengue or Zika viruses (Gootenberg et al. 2018). Future studies should investigate the collateral cleavage trans-activity properties of other cas12 proteins that can detect multiplex pathogens in one-pot cas12 assay from the different color labels on the reporter.

The RPA-assisted cas12a assay using both gRNA\_E and gRNA\_A had a limit of detection of 100 copies of DNA target per reaction. The detection limit of the RPA-assisted cas12a assay using gRNA\_E and gRNA\_A compared to gel electrophoresis detection was 1,000 and 100 times greater, respectively. Furthermore, the RPA products yielded the expected amplicons and generated primer dimerization or non-specific smear bands that appeared on gel electrophoresis, leading to the interference of detection but unaffected by cas12a detection. This was unsurprising because signal amplification from the RPA reaction was initiated by the operation of the cas12a-gRNA complex for DNA target binding. Therefore, cas12a collaborated with the isothermal amplification methods extensively used for detection such as LAMP-cas12a for African swine fever and *Escherichia coli* detection (Lee and Oh 2022; Yang et al. 2022). RPA-cas12a for COVID-19 and *Toxoplasma gondii* detection (Ding et al. 2020; Lei et al. 2021). The sensitivity of the assay was dependent on (i) the amplicon the size of RPA (the smaller amplicon size, the more sensitive) and (ii) the condition of the cas12a assay such as the concentration of cas and gRNA used, the sequences of the reporter and the additives (Lv et al. 2021). When the result of RPA coupled with cas12a was compared with gel electrophoresis detection, the RPA-assisted cas12a assay had greater specificity and sensitivity. The limit of detection could be improved using high-efficiency ssDNA reporters to produce the fluorescence signal. A study of engineering the DNA reporter (TTATT-5C) enabled a ten-fold sensitivity increase compared with the TTATT reporter, while the optimal concentration of DNA reporter engineering clearly showed a fluorescence signal between the target and non-target in the cas12a assay (Lee et al. 2022). This study determined the background of fluorescence signals in the negative control that generated a fluorescence signal even if the DNA target disappeared. In future studies, a new ssDNA reporter should be modified to improve the performance of cas12a for trans-activity detection.

Diagnostics for rickettsial agents provide different detection efficiencies. Microscopic observation has been used for rickettsia diagnosis with low sensitivity due to difficulty in observing blood smears when dogs become infected after two weeks (Rucksaken et al. 2019). The ELISA used for detecting rickettsial agents reported high sensitivity, with detection 14 days after inoculation as in the early infection,

the amount of antibody produced is limited to detect, while molecular assay gave detection within seven days and the serological method gave a false positive result (Waner et al. 2000; Suksawat et al. 2000). Currently, the diagnosis of rickettsia includes conventional PCR or real-time PCR. Real-time PCR for *Ehrlichia* species has limited detection at 50 and 100 copies per reaction (Cárdenas et al. 2007; Lloyd et al. 2011). Our previous study demonstrated that the detection limit of real-time PCR based on multiplex HRM using Bact16S-A for *E. canis* and *A. platys* was  $10^3$  copy number (Buddhachat et al. 2020). RPA-assisted cas12a using EA\_16S was ten times more sensitive, with a detection limit of  $10^2$  copy number.

The RPA-assisted cas12a assay using gRNA\_E and gRNA\_A was validated with real clinical samples that showed efficiency for *E. canis* and *A. platys* detection from canine peripheral blood. However, the results obtained from HRM might give the deviation of melting temperature from melting peak of *E. canis* and *A. platys* which it is likely to be other closely related species of them such as *E. chaffeensis*, *E. ewingii* and *A. phagocytophilum* (Supplemental Figure S8). The RPA enabled DNA amplification of *E. canis* and *A. platys*, and both gRNA\_E and gRNA\_A provided high accuracy to detect pathogens owing to disappearing fluorescence signals in non-target DNA. The fluorescence signals acquired from the RPA-assisted cas12a assay could be readily observed by the naked eye under a blue LED transilluminator. The detection limit of this method was sufficient for real sample detection. Moreover, the RPA-assisted cas12a assay results gave precision and accuracy with the HRM assay of more than 75% for *E. canis* and *A. platys* detection. Eleven clinical samples exhibited positive for cas12a assay but negative for HRM, indicating that the RPA-assisted cas12a assay showed superior ability to discriminate *Ehrlichia* and *Anaplasma* over the HRM assay. In the case of coinfection, a large difference in the two melting peaks obtained from the two pathogens can dominate a minor melting peak, leading to difficulty or impossibility to detect their presence. We also confirmed the results obtained from both approaches by the sequence similarity to *E. canis*, with more than 97% identity. However, a low success rate of sequencing (39% (7/18 samples)) was observed, possibly resulting from the admixture of two amplicons caused by coinfection and some samples without positive amplicons (e.g. 640208 and 640501) (Supplemental Figure S9). Furthermore, RPA-assisted cas12a assay using gRNA\_A gave a false positive for 640208 from the point mutation on the DNA target for gRNA at the seed region due to the presence of one different base (G/A) (Supplemental Figure S2A). Based on multiple alignments, the EA\_16S primer enabled DNA amplification for both *Ehrlichia* and *Anaplasma* genera, whereas using cas12a with gRNA\_A or gRNA\_E facilitated discrimination between *Ehrlichia* and *Anaplasma* genera (Supplemental

Figure S2A). These results indicated the moderate reliability of both methods to detect *E. canis* and *A. platys* in real blood samples. Diagnosis of the presence of pathogens in canine blood diseases should be performed rapidly and accurately for precise and effective treatment of the ailments, and this approach can detect *E. canis* and *A. platys* within two hours. Moreover, fast diagnosis and treatment will assist in saving the dog's life and decrease contagion of the disease (Lauzi et al. 2016; Yuasa et al. 2017). This study articulated the optimal situation of the RPA-assisted cas12a assay to detect pathogens in canine blood with specificity and sensitivity. Moreover, the method was simple and detection was fast, requiring few instruments with easy-to-interpret results. The lateral flow dipstick can be useful to interpret results for detection in the field (Xu et al. 2022; Zhang et al. 2021). However, pathogen diagnostics in the field applying the RPA-assisted cas12a assay might have airborne contamination. Therefore, the hands-on process needs to be performed carefully and the reaction should be performed in a closed area. When using the RPA-assisted cas12a assay for practical POCT, efficient and rapid DNA extraction from canine blood needs to be developed such as using a dipstick, buffer or heating methods. Simple DNA extraction methods can be directly input into the RPA reaction and detected by the cas12a assay (Bereczky et al. 2005; Koontz et al. 2019; Mason and Botella 2020).

## Conclusions

Results demonstrated that the RPA-assisted cas12a assay can be implemented for *E. canis* and *A. platys* in canine blood, with high specificity and sensitivity detection relative to a conventional or real-time PCR. The detection process is simple, fast and has few steps. The method is appropriate to use in the field because it requires minimal resources and instruments. This technique can be implemented in low-resource areas to detect pathogens in the blood of homeless dogs in countries with limited resources. This method can improve multiplex in a one-pot platform that utilizes coinfection agent detection with reduction in contamination. We believe that an efficient diagnosis is an important tool that can decrease and restrain parasitic diseases. Moreover, The RPA-assisted cas12a assay as a novel, fast and inexpensive method can assist the veterinarian for the precision treatment of infected dogs.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11259-023-10114-0>.

**Acknowledgements** This project was funded by the National Research Council of Thailand (NRCT) (Grant No. N42A650332) and the Faculty of Science, Naresuan University (Grant No. R2565A060). Additional funding was supported by the Global and Frontier Research University

Fund, Naresuan University; Grant number R2566C051 and the Excellence Center in Veterinary Bioscience, Chiang Mai University, Chiang Mai, Thailand. We also thank the Animal Molecular Diagnostic Service (AMDS), Chiang Mai, Thailand for providing the DNA clinical samples.

**Author contributions** Kittisak Buddhachat (K.B.) conceptualized the study, conducted the experiments and performed funding acquisition and project administration. Supharn Paenkaew (S.P.) conducted all the experiments and wrote the first draft of the manuscript. Waranee Pradit (W.P.) and Puntita Siengdee (P.S.) collected canine blood specimens and performed DNA isolation. Nongluck Jaito (N.J.), Siriwardee Chomdej (S.C.) and Korakot Nganvongpanit (K.N.) provided suggestions. K.B. and S.P. analyzed the statistical data and wrote the manuscript. All the authors have read and approved the final version of the manuscript.

**Funding** National Research Council of Thailand (NRCT) (Grant No. N42A650332).

Faculty of Science, Naresuan University (Grant No. R2565A060). Global and Frontier Research University Fund, Naresuan University; Grant number R2566C051.

Excellence Center in Veterinary Bioscience, Chiang Mai University, Chiang Mai, Thailand.

**Data availability** Data of LoD determination were explained in Supplemental file 1 HRM results were presented in Supplemental File 2, with sequences obtained from this study displayed in Supplemental File 3. All Supplemental Figures was illustrated in Supplemental file 4.

## Declarations

**Competing interests** The authors declare no competing interests.

**Ethical approval** DNA samples were provided by AMDS, and this was confirmed by the Animal Ethics Committee, Center of Animal Research, Naresuan University (NUCAR) (License number NU-AEE620503).

**Consent to participate** Informed consent was obtained from all study participants.

**Consent to publish** The authors hereby consent to publication of this study paper.

**Conflict of interest** All authors declare no conflicts of interest associated with this publication.

## References

- Almazán C, González-Álvarez VH, Fernández de Mera IG, Cabezas-Cruz A, Rodríguez-Martínez R, De la Fuente J (2016) Molecular identification and characterization of *Anaplasma platys* and *Ehrlichia canis* in dogs in Mexico. *Ticks Tick Borne Dis* 7:276–283. <https://doi.org/10.1016/j.ttbdis.2015.11.002>
- Aman R, Mahas A, Marsic T, Hassan N, Mahfouz MM (2020) Efficient, rapid, and sensitive detection of plant RNA viruses with one-pot RT-RPA–CRISPR/Cas12a assay. *Front Microbiol* 11:610872. <https://doi.org/10.3389/fmicb.2020.610872>
- Armbruster DA, Pry T (2008) Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* 29:S49–S52
- Berezky S, Mårtensson A, Gil JP, Färnert A (2005) Short report: rapid DNA extraction from archive blood spots on filter paper

- for genotyping of *Plasmodium falciparum*. *Am J Trop Med Hyg* 72:249–251. <https://doi.org/10.4269/ajtmh.2005.72.249>
- Buddhachat K, Meerod T, Pradit W, Siengdee P, Chomdej S, Nganvongpanit K (2020) Simultaneous differential detection of canine blood parasites: multiplex high-resolution melting analysis (mHRM). *Ticks Tick Borne Dis* 11:101370. <https://doi.org/10.1016/j.ttbdis.2020.101370>
- Buddhachat K, Paenkaew S, Sriparoj N, Gupta YM, Pradit W, Chomdej S (2021) Bar-cas12a, a novel and rapid method for plant species authentication in case of *Phyllanthus amarus* Schumacher & Thonn. *Sci Rep* 11:20888. <https://doi.org/10.1038/s41598-021-00006-1>
- Buddhachat K, Sriparoj N, Ritbamrung O, Inthima P, Ratanasut K, Boonsrangsom T, Rungrat T, Pongcharoen P, Sujipuli K (2022) RPA-assisted cas12a system for detecting pathogenic *Xanthomonas oryzae*, a causative agent for bacterial leaf blight disease in rice. *Rice Sci* 29:340–352. <https://doi.org/10.1016/j.rsci.2021.11.005>
- Cárdenas AM, Doyle CK, Zhang X, Nethery K, Corstvet RE, Walker DH, McBride JW (2007) Enzyme-linked immunosorbent assay with conserved immunoreactive glycoproteins gp36 and gp19 has enhanced sensitivity and provides species-specific immunodiagnosis of *Ehrlichia canis* infection. *Clin Vaccine Immunol* 14:123–128. <https://doi.org/10.1128/CLV.00361-06>
- Cardoso L, Tuna J, Vieira L, Yisaschar-Mekuzas Y, Baneth G (2010) Molecular detection of *Anaplasma platys* and *Ehrlichia canis* in dogs from the north of Portugal. *Vet J* 183:232–233. <https://doi.org/10.1016/j.tvjl.2008.10.009>
- Chaijarasphong T, Thammachai T, Itsathitphaisarn O, Sritunyaluksana K, Suebsing R (2019) Potential application of CRISPR-Cas12a fluorescence assay coupled with rapid nucleic acid amplification for detection of white spot syndrome virus in shrimp. *Aquac* 512:734340. <https://doi.org/10.1016/j.aquaculture.2019.734340>
- Chandrasekaran SS et al (2022) Rapid detection of SARS-CoV-2 RNA in saliva via cas13. *Nat Biomed Eng* 6:944–956. <https://doi.org/10.1038/s41551-022-00917-y>
- Chen JS, Ma E, Harrington LB, da Costa M, Tian X, Palefsky JM, Doudna JA (2018) CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science* 360:436–439. <https://doi.org/10.1126/science.aar6245>
- Chomel B (2011) Tick-borne infections in dogs—an emerging infectious threat. *Vet Parasitol* 179:294–301. <https://doi.org/10.1016/j.vetpar.2011.03.040>
- Cicuttin GL, Brambati DF, Rodríguez Eugui JI, Lebrero CG, de Salvo MN, Beltrán FJ, Gury Dohmen FE, Jado I, Anda P (2014) Molecular characterization of *Rickettsia massiliae* and *Anaplasma platys* infecting *Rhipicephalus sanguineus* ticks and domestic dogs, Buenos Aires (Argentina). *Ticks Tick Borne Dis* 5:484–488. <https://doi.org/10.1016/j.ttbdis.2014.03.001>
- Crannell ZA, Rohrman B, Richards-Kortum R (2014) Equipment-free incubation of recombinase polymerase amplification reactions using body heat. *PLoS One* 9:e112146. <https://doi.org/10.1371/journal.pone.0112146>
- Ding X, Yin K, Li Z, Lalla RV, Ballesteros E, Sfeir MM, Liu C (2020) Ultrasensitive and visual detection of SARS-CoV-2 using all-in-one dual CRISPR-Cas12a assay. *Nat Commun* 11:4711. <https://doi.org/10.1038/s41467-020-18575-6>
- Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Rurangirwa FR (2001) Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and “HGE agent” as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 51:2145–2165. <https://doi.org/10.1099/00207173-51-6-2145>

- Fourie JJ, Stanneck D, Luus HG, Beugnet F, Wijnveld M, Jongejan F (2013) Transmission of *Ehrlichia canis* by *Rhipicephalus sanguineus* ticks feeding on dogs and on artificial membranes. *Vet Parasitol* 197:595–603. <https://doi.org/10.1016/j.vetpar.2013.07.026>
- Fu S, Qu G, Guo S, Ma L, Zhang N, Zhang S, Gao S, Shen Z (2011) Applications of loop-mediated isothermal DNA amplification. *Appl Biochem Biotechnol* 163:845–850. <https://doi.org/10.1007/s12010-010-9088-8>
- Gerashchenkov GA, Rozhnova NA, Kuluev BR, Kiryanova O, Yu GGR, Knyazev AV, Vershinina ZR, Mikhailova EV, Chemeris DA, Matniyazov RT, Baimiev AK, Gubaidullin IM, Baimiev AK, Chemeris AV (2020) Design of guide RNA for CRISPR/Cas plant genome editing. *Mol Biol* 54:24–42. <https://doi.org/10.1134/s0026893320010069>
- Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ, Zhang F (2018) Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a and Csm6. *Science* 360:439–444. <https://doi.org/10.1126/science.aag0179>
- Gulati G, Song J, Florea AD, Gong J (2013) Purpose and criteria for blood smear scan, blood smear examination, and blood smear review. *Ann Lab Med* 33:1–7. <https://doi.org/10.3343/alm.2013.33.1.1>
- Harrus S, Waner T (2011) Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*): an overview. *Vet J* 187:292–296. <https://doi.org/10.1016/j.tvjl.2010.02.001>
- Inokuma H, Fujii K, Matsumoto K, Okuda M, Nakagome K, Kosugi R, Hirakawa M, Onishi T (2002) Demonstration of *Anaplasma (Ehrlichia) platys* inclusions in peripheral blood platelets of a dog in Japan. *Vet Parasitol* 110:145–152. [https://doi.org/10.1016/s0304-4017\(02\)00289-3](https://doi.org/10.1016/s0304-4017(02)00289-3)
- Kham-Kjing N, Ngo-Giang-huong N, Tragoolpua K, Khamduang W, Hongjaisee S (2022) Highly specific and rapid detection of hepatitis c virus using RT-LAMP-coupled CRISPR–Cas12 assay. *Diagnostics* 12:1524. <https://doi.org/10.3390/diagnostics12071524>
- Kim S, Ji S, Koh HR (2021) CRISPR as a diagnostic tool. *Biomolecules* 11:1162. <https://doi.org/10.3390/biom11081162>
- Koontz D, Dollard S, Cordovado S (2019) Evaluation of rapid and sensitive DNA extraction methods for detection of cytomegalovirus in dried blood spots. *J Virol Methods* 265:117–120. <https://doi.org/10.1016/j.jviromet.2019.01.005>
- Kramer MF, Coen DM (2001) Enzymatic amplification of DNA by PCR: standard procedures and optimization. *Curr Protoc Mol Biol* 56:1511–15114. <https://doi.org/10.1002/0471142727.mb1501s56>
- Lauzi S, Maia JP, Epis S, Marcos R, Pereira C, Luzzago C, Santos M, Puente-Payo P, Giordano A, Pajoro M, Sironi G, Faustino A (2016) Molecular detection of *Anaplasma platys*, *Ehrlichia canis*, *Hepatozoon canis* and *Rickettsia monacensis* in dogs from Maio Island of Cape Verde archipelago. *Ticks Tick Borne Dis* 7:964–969. <https://doi.org/10.1016/j.ttbdis.2016.05.001>
- Lee SY, Oh SW (2022) Filtration-based LAMP-CRISPR/Cas12a system for the rapid, sensitive and visualized detection of *Escherichia coli* O157:H7. *Talanta* 241:123186. <https://doi.org/10.1016/j.talanta.2021.123186>
- Lee S, Nam D, Park JS, Kim S, Lee ES, Cha BS, Park KS (2022) Highly efficient DNA reporter for CRISPR/Cas12a-based specific and sensitive biosensor. *Biochip J* 13:1–8. <https://doi.org/10.1007/s13206-022-00081-0>
- Lei R, Li L, Wu P, Fei X, Zhang Y, Wang J, Zhang D, Zhang Q, Yang N, Wang X (2021) RPA/CRISPR/Cas12a-based on-site and rapid nucleic acid detection of *Toxoplasma gondii* in the environment. *ACS Synth Biol* 11:1772–1781. <https://doi.org/10.1021/acssynbio.1c00620>
- Li J, Macdonald J (2015) Advances in isothermal amplification: novel strategies inspired by biological processes. *Biosens Bioelectron* 64:196–211. <https://doi.org/10.1016/j.bios.2014.08.069>
- Li L, Li S, Wu N, Wu J, Wang G, Zhao G, Wang J (2019) HOLMESv2: A CRISPR-Cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation. *ACS Synth Biol* 8:2228–2237. <https://doi.org/10.1021/acssynbio.9b00209>
- Lloyd SJ, LaPatra SE, Snekvik KR, Cain KD, Call DR (2011) Quantitative PCR demonstrates a positive correlation between a Rickettsia-like organism and severity of strawberry disease lesions in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis* 34:701–709. <https://doi.org/10.1111/j.1365-2761.2011.01285.x>
- Lu S, Li F, Chen Q, Wu J, Duan J, Lei X, Zhang Y, Zhao D, Bu Z, Yin H (2020) Rapid detection of African swine fever virus using Cas12a-based portable paper diagnostics. *Cell Discov* 6:18. <https://doi.org/10.1038/s41421-020-0151-5>
- Lv H, Wang J, Zhang J, Chen Y, Yin L, Jin D, Gu D, Zhao H, Xu Y, Wang J (2021) Definition of CRISPR Cas12a trans-cleavage units to facilitate CRISPR diagnostics. *Front Microbiol* 12:766464. <https://doi.org/10.3389/fmicb.2021.766464>
- Mason MG, Botella JR (2022) Rapid (30-second), equipment-free purification of nucleic acids using easy-to-make dipsticks. *Nat Protoc* 15:3663–3677. <https://doi.org/10.1038/s41596-020-0392-7>
- Mukama O, Yuan T, He Z, Li Z, Habimana JD, Hussain M, Li W, Yi Z, Liang Q, Zeng L (2020) A high fidelity CRISPR/Cas12a based lateral flow biosensor for the detection of HPV16 and HPV18. *Sens Actuator A Phys* 316:128119. <https://doi.org/10.1016/j.snb.2020.128119>
- Mylonakis ME, Koutinas AF, Billinis C, Leontides LS, Kontos V, Papadopoulos O, Rallis T, Fytianou A (2003) Evaluation of cytology in the diagnosis of acute canine monocytic ehrlichiosis (*Ehrlichia canis*): a comparison between five methods. *Vet Microbiol* 91:197–204. [https://doi.org/10.1016/s0378-1135\(02\)00298-5](https://doi.org/10.1016/s0378-1135(02)00298-5)
- Nasr A, Ghafar M, Elhariri M (2020) Detection of *Anaplasma platys* and *Ehrlichia canis* in *Rhipicephalus sanguineus* ticks attached to dogs from Egypt; a public health concern. *J Vet Med* 66:1–9. <https://doi.org/10.21608/vmjg.2020.157540>
- Pérez-Macchi S, Pedrozo R, Bittencourt P, Müller A (2019) Prevalence, molecular characterization and risk factor analysis of *Ehrlichia canis* and *Anaplasma platys* in domestic dogs from Paraguay. *Comp Immunol Microbiol Infect Dis* 62:31–39. <https://doi.org/10.1016/j.cimid.2018.11.015>
- Pinyowong D, Jittapalpong S, Suksawat F, Stich RW, Thamchaipenet A (2008) Molecular characterization of Thai *Ehrlichia canis* and *Anaplasma platys* strains detected in dogs. *Infect Genet Evol* 8:433–438. <https://doi.org/10.1016/j.meegid.2007.06.002>
- Qin P, Park M, Alfson KJ, Tamhankar M, Carrion R, Patterson JL, Griffiths A, He Q, Yildiz A, Mathies R, Du K (2019) Rapid and fully microfluidic ebola virus detection with CRISPR–Cas13a. *ACS Sens* 4:1048–1054. <https://doi.org/10.1021/acssensors.9b00239>
- Rohrman BA, Leautaud V, Molyneux E, Richards-Kortum RR (2012) A lateral flow assay for quantitative detection of amplified HIV-1 RNA. *PLoS ONE* 7:0045611. <https://doi.org/10.1371/journal.pone.0045611>
- Rucksaken R, Maneeruttanarungroj C, Maswanna T, Sussadee M, Kanbutra P (2019) Comparison of conventional polymerase chain reaction and routine blood smear for the detection of *Babesia canis*, *Hepatozoon canis*, *Ehrlichia canis*, and *Anaplasma platys* in Buriram Province, Thailand. *Vet World* 12:700–705. <https://doi.org/10.14202/vetworld.2019.700-705>
- Suksawat J, Hegarty BC, Breitschwerdt EB (2000) Seroprevalence of *Ehrlichia canis*, *Ehrlichia equi*, and *Ehrlichia risticii* in sick dogs from north Carolina and Virginia. *J Vet Intern Med* 14:50–55. <https://doi.org/10.1111/j.1939-1676.2000.tb01499.x>
- Unver A, Rikihisa Y, Kawahara M, Yamamoto S (2003) Analysis of 16S rRNA gene sequences of *Ehrlichia canis*, *Anaplasma platys*, and *Wolbachia* species from canine blood in Japan. *In Ann N Y Acad Sci* 990:692–698. <https://doi.org/10.1111/j.1749-6632.2003.tb07445.x>

- Waner T, Strenger C, Keysary A (2000) Comparison of a clinic-based ELISA test kit with the immunofluorescence test for the assay of *Ehrlichia canis* antibodies in dogs. *J Vet Diagn Invest* 12:101370. <https://doi.org/10.1016/j.tbdis.2020.101370>
- Wang B, Wang R, Wang D, Wu J, Li J, Wang J, Liu H, Wang Y (2019) Cas12aVDeT: A CRISPR/Cas12a-Based Platform for Rapid and Visual Nucleic Acid Detection. *Anal Chem* 91:12156–12161. <https://doi.org/10.1021/acs.analchem.9b01526>
- Wang X, Ji P, Fan H, Dang L, Wan W, Liu S, Li Y, Yu W, Li X, Ma X, Ma X, Zhao Q, Huang X, Liao M (2020) CRISPR/Cas12a technology combined with immunochromatographic strips for portable detection of African swine fever virus. *Commun Biol* 3:62. <https://doi.org/10.1038/s42003-020-0796-5>
- Xu H, Tang H, Li R, Xia Z, Yang W, Zhu Y, Liu Z, Lu G, Ni S, Shen J (2022) A new method based on LAMP-CRISPR–Cas12a-lateral flow immunochromatographic strip for detection. *Infect Drug Resist* 15:685–696. <https://doi.org/10.2147/IDR.S348456>
- Yabsley MJ, McKibben J, Macpherson CN, Cattan PF, Cherry NA, Hegarty BC, Breitschwerdt EB, O'Connor T, Chandrashekar R, Paterson T, Perea ML, Ball G, Friesen S, Goedde J, Henderson B, Sylvester W (2008) Prevalence of *Ehrlichia canis*, *Anaplasma platys*, *Babesia canis vogeli*, *Hepatozoon canis*, *Bartonella vinsonii berkhoffii*, and *Rickettsia* spp. in dogs from Grenada. *Vet Parasitol* 151:279–285. <https://doi.org/10.1016/j.vetpar.2007.11.008>
- Yang B, Shi Z, Ma Y, Wang L, Cao L, Luo J, Wan Y, Song R, Yan Y, Yuan K, Tian H, Zheng H (2022) LAMP assay coupled with CRISPR/Cas12a system for portable detection of African swine fever virus. *Transbound Emerg Dis* 69:e216–e223. <https://doi.org/10.1111/tbed.14285>
- Yuasa Y, Tsai YL, Chang CC, Hsu TH, Chou CC (2017) The prevalence of *Anaplasma platys* and a potential novel *Anaplasma* species exceed that of *Ehrlichia canis* in asymptomatic dogs and *Rhipicephalus sanguineus* in Taiwan. *J Vet Med* 79:1494–1502. <https://doi.org/10.1292/jvms.17-0224>
- Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van der Oost J, Regev A, Koonin EV, Zhang F (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163:759–771. <https://doi.org/10.1016/j.cell.2015.09.038>
- Zhang C, Li Z, Chen M, Hu Z, Wu L, Zhou M, Liang D (2021) Cas12a and lateral flow strip-based test for rapid and ultrasensitive detection of spinal muscular atrophy. *Biosensors* 11:154. <https://doi.org/10.3390/bios11050154>

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.