Development of a Sensitive and Rapid Recombinase Polymerase Amplification Assay for Detection of *Anaplasma phagocytophilum*

Le Jiang,^a Philip Ching,^b Chien-Chung Chao,^{a,c} J. Stephen Dumler,^c Wei-Mei Ching^{a,c,†}

^aViral and Rickettsial Diseases Department, Infectious Diseases Directorate, Naval Medical Research Center, Silver Spring, Maryland, USA

^bAplix Research Inc., North Potomac, Maryland, USA

^cUniformed Services University of the Health Sciences, Bethesda, Maryland, USA

ABSTRACT Human granulocytic anaplasmosis (HGA) is a tick-borne disease caused by the obligate intracellular Gram-negative bacterium *Anaplasma phagocytophilum*. The disease often presents with nonspecific symptoms with negative serology during the acute phase. Direct pathogen detection is the best approach for early confirmatory diagnosis. Over the years, PCR-based molecular detection methods have been developed, but optimal sensitivity is not achieved by conventional PCR while real-time PCR requires expensive and sophisticated instruments. To improve the sensitivity and also develop an assay that can be used in resource-limited areas, an isothermal DNA amplification assay based on recombinase polymerase amplification (RPA) was developed. To do this, we identified a 171-bp DNA sequence within multiple paralogous copies of *msp2* within the genome of *A. phagocytophilum*. Our novel RPA assay targeting this sequence has an analytical limit of detection of one genome equivalent copy of *A. phagocytophilum* and can reliably detect 125 bacteria/ml in human blood. A high level of specificity was demonstrated by the absence of nonspecific amplification using genomic DNA from human or DNA from other closely-related pathogenic bacteria, such as *Anaplasma platys*, *Ehrlichia chaffeensis*, *Orientia tsutsugamushi*, and *Rickettsia rickettsii*, etc. When applied to patient DNA extracted from whole blood, this new RPA assay was able to detect 100% of previously diagnosed *A. phagocytophilum* cases. The sensitivity and rapidness of this assay represents a major improvement for early diagnosis of *A. phagocytophilum* in human patients and suggest a role for better surveillance in its reservoirs or vectors, especially in remote regions where resources are limited.

KEYWORDS *Anaplasma phagocytophilum*, recombinase polymerase amplification (RPA), multicopy DNA, rapid detection

Anaplasma phagocytophilum is an obligate intracellular Gram-negative bacterium that can be transmitted to humans and domestic animals mainly through *Ixodes* ticks present in the Northern Hemisphere (1). Its infection causes tick-borne fever (TBF) in domestic animals and human granulocytic anaplasmosis (HGA) in patients. As a multihost pathogen, *A. phagocytophilum* puts significant economic burden on livestock production and increases health risks for human and their pets as well.

Clinical diagnosis of HGA is challenging, as many patients present with nonspecific symptoms and signs including fever, malaise, headache, and myalgia, etc. (2). This often delays antibiotic treatment, predominantly doxycycline, which is most effective during the early course of the infection. Traditionally, peripheral blood smears are examined microscopically, and the presence of morulae in the cytoplasm of neutrophils can be used for diagnosis during the first week of illness (3). However, this method might be

Citation Jiang L, Ching P, Chao C-C, Dumler JS, Ching W-M. 2020. Development of a sensitive and rapid recombinase polymerase amplification assay for detection of *Anaplasma phagocytophilum*. J Clin Microbiol 58:e01777-19. <https://doi.org/10.1128/JCM.01777-19>.

Editor Karen C. Carroll, Johns Hopkins University School of Medicine

This is a work of the U.S. Government and is not subject to copyright protection in the United States. Foreign copyrights may apply.

Address correspondence to Chien-Chung Chao, chien-chung.chao.civ@mail.mil.

† Deceased 20 February 2019.

Received 23 October 2019

Returned for modification 17 November 2019

Accepted 25 February 2020

Accepted manuscript posted online 4 March 2020

Published 23 April 2020

error-prone in cases of low-level bacteremia or due to other inclusions or cytoplasmic granules. Serology-based clinical tests, such as immunofluorescent assay (IFA) have been useful, but they require the presence of *Anaplasma*-specific antibodies, which are not detectable until the second week after infection. Furthermore, cross-reactions with other *Anaplasma* species or closely related bacterial species, such as *Ehrlichia chaffeensis*, are possible. Another drawback of the aforementioned methods is that they do not offer direct pathogen detection in invertebrates, such as its tick vectors for prevalence or surveillance studies. DNA-based molecular detection has long been used for identification of *Anaplasma* species and offers much higher levels of sensitivity and specificity. For example, DNA sequences within *rrs* (4), *msh2* (5), and *msh4* (6) genes, among others, have been used for conventional or real-time PCR assays for *A. phagocytophilum* detection. However, PCR-based direct pathogen detection requires well-trained technicians and expensive equipment, which are usually not readily available in areas with limited resources.

To avoid reliance on thermal cyclers, several technologies to amplify nucleic acids under isothermal conditions have been developed, including loop-mediated isothermal amplification (LAMP), rolling cycle amplification (RCA), helicase-dependent amplification (HAD), and recombinase polymerase amplification (RPA). Each technology has its own strength/weakness and differs in terms of mechanism of amplification, operating temperature, and target requirement (7). RPA assay was developed as a novel method to efficiently amplify DNA at low-temperature conditions (between 37 and 42°C), thus providing a simple alternative for nucleic acid detection (8). It amplifies double-stranded DNA sequences using recombinase, DNA polymerase, and DNA-binding proteins and has been successfully used to detect bacterial pathogen DNA (9–11). When coupled with a reverse transcriptase, it can also effectively detect RNA viruses (12, 13). In the present study, we developed a rapid and sensitive RPA assay for detecting *A. phagocytophilum* based on a multicopy DNA fragment. It is highly sensitive and specific and has the potential to be utilized as a point-of-care diagnostic tool in resource-constrained regions.

MATERIALS AND METHODS

Sequence analysis of *A. phagocytophilum*. The whole-genome sequence of *A. phagocytophilum* (HZ strain) was downloaded from the NCBI database (GenBank accession number [NC_007797.1](#)). A 171-bp DNA fragment within *msh2* was found to have 16 copies using a sequence analysis software developed by Aplix Research, Inc. (North Potomac, MD). Genomic locations containing the 171-bp sequences are as follows: 173581 to 173751, 175187 to 175357, 1173958 to 1174128, 1180574 to 1180744, 1227961 to 1228131, 1236089 to 1236259, 1244562 to 1244732, 1299262 to 1299432, 1312928 to 1313098, 1321661 to 1321831, 1337699 to 1337869, 1341524 to 1341694, 1343995 to 1344165, 1354477 to 1354647, 1381117 to 1381287, 1459403 to 1459573.

Primer and probe design. Forward and reverse primers for RPA assay were designed using Primer3 software (version 0.4.0) (14) and manually extended in the 5' direction to 30 bp in length. Primers for real-time PCR were designed based on the same 171-bp region using the online Assay Design Center on the Roche website. All primers were synthesized by Eurofins Genomics (Louisville, KY). A fluorescence-labeled exo probe was designed according to the manual from TwistDx (Cambridge, United Kingdom) and synthesized by LGC Biosearch Technologies (Petaluma, CA). All primer/probe sequences used in this study are listed in Table 1. Primer-BLAST (15) was used to evaluate specificity of chosen primer sets against RefSeq representative genome database related to bacteria, viruses, ticks, and human.

DNA sources, preparation, and quantification. *Ehrlichia chaffeensis* (Liberty strain) DNA was provided by BEI Resources (Manassas, VA). *Borrelia burgdorferi* (B31 strain) DNA was from ATCC (Manassas, VA). DNA for *Anaplasma platys* was a kind gift from J. Stephen Dumler. DNA for *Orientia tsutsugamushi* (Karp strain) and several *rickettsia* species were extracted from cultured bacteria purified via Renografin gradients and previously stored in the lab. *A. phagocytophilum* (Webster strain) was grown in human HL-60 cells. The culture was harvested and stored in liquid nitrogen when the number of bacteria reached about 50 to 100 bacteria per cell. After thawing, DNA extraction was performed using Qiagen DNA minikit (Germantown, MD) following manufacturer's protocol for Gram-negative bacteria. DNA absorbance was measured on a NanoDrop 2000 spectrophotometer. Genome equivalent (GE) copy number of *A. phagocytophilum* was quantified by a standard curve generated from serial dilutions of a reference plasmid containing a fragment from a single-copy *ankA* gene using real-time PCR. To prepare the DNA samples used in Fig. 2C and D, various GE copy numbers of *A. phagocytophilum* DNA (250, 25, 5, and 0) were spiked into 200 μ l of normal human blood. This was followed by DNA extraction using the Qiagen DNA minikit, and the whole process of spike-in and DNA extraction was repeated three times. DNA from each extraction was eluted in 20 μ l elution buffer, 4 μ l of which was used for quantitative real-time PCR (qPCR) or RPA assay (2 to 7 reactions were performed for each level of copy number).

TABLE 1 RPA and qPCR primers (5' to 3' direction) used in this study

Primer name	Primer sequence	Reference
AnaplasmaRPA_F1	TCTAATACCCTTGGTCTTGAAGCGCTCGTA	This article
AnaplasmaRPA_F2	TGGTCTTGAAGCGCTCGTAACCAATCTCAA	This article
AnaplasmaRPA_F3	CTCGTAACCAATCTCAAGCTCAACCCTGGC	This article
AnaplasmaRPA_R1	CATGCTTGTAGCTATGGAAGGCAGTGTGG	This article
AnaplasmaRPA_R2	CTGATCCTCGGATTGGGTTAAGGACAACA	This article
AnaplasmaRPA_R3	TCCTCGGATTGGGTTAAGGACAACATGCT	This article
Anaplasma exo probe	AATCTCAAGCTCAACCCTGGCACCACCAA[T(FAM)]AC[dSpacer]A[T(BHQ-1)] AACCAACTGCCTTC-[SpacerC3]	This article
msp2F	GTCTTGAAGCGCTCGTAACC	This article
msp2R	GCTTGTAGCTATGGAAGGCAGT	This article
ankA-F	CAGTCGTGAATGTAGAGGGAAAAAC	Dong et al., 2013 (24)
ankA-R	GGAATCCCCCTTCAGGAACCTTG	Dong et al., 2013 (24)
ApMSP2f	ATGGAAGGTAGTGTGGTTATGGTATT	Courtney et al., 2004 (5)
ApMSP2r	TTGGTCTTGAAGCGCTCGTA	Courtney et al., 2004 (5)

PCR, cloning, and real-time PCR. PCR was performed using Platinum PCR SuperMix high fidelity from Thermo Fisher Scientific (Waltham, MA) according to manufacturer's instructions. Initial evaluation of RPA primer sets was carried out in a PCR thermal cycler for 18 cycles (95°C, 20 s; 64°C, 20 s; and 68°C, 40 s) followed by agarose gel electrophoresis. To generate plasmids for standard curve and determination of limit of detection, DNA fragments for *ankA* (primer set ankA-F/ankA-R; Table 1) and *msp2* (primer set AnaplasmaRPA_F1/AnaplasmaRPA_R2; Table 1) were amplified from *A. phagocytophilum* genomic DNA (Webster strain) for 18 and 16 cycles, respectively, followed by immediate PCR purification and TOPO cloning into the pCR-XL-TOPO vector (Thermo Fisher Scientific). Quantitative real-time PCR was performed using QuantiFast SYBR green PCR kit (Qiagen) on a 7500 fast real-time PCR system (Applied Biosystems) with a standard 40-cycle amplification protocol.

RPA reactions. Reagents for RPA were provided in the TwistAmp exo kit (TwistDx), and RPA reactions were performed according to the manufacturer's instruction. Briefly, a 47.5- μ l mixture containing 29.5 μ l rehydration buffer, 300 nM each primer (Anaplasma RPA_F3/Anaplasma RPA_R3), 120 nM probe, and DNA template (2 to 10 μ l) was added and mixed with lyophilized RPA enzymes. After adding 2.5 μ l of magnesium acetate (MgAc, 280 mM) to start the reaction, the 8-tube reaction strip was immediately mixed and placed in Twista tube scanner instrument (TwistDx) for incubation at 39°C. Four minutes after the start of the reaction, the strip was quickly removed and mixed one more time before incubation at 39°C for another 16 min. Fluorescence signal was monitored and analyzed in the Twista studio software.

Clinical samples. Human blood samples and/or DNA from patients with *A. phagocytophilum* or *E. chaffeensis* infection were stored frozen at -80°C until used. Their acquisition and use were approved through human subject protocols at Johns Hopkins Medicine (Baltimore, MD), University of Maryland, Baltimore, or the St. Mary's/Duluth Clinic (Duluth, MN) institutional review boards (IRBs). The final diagnosis was based on the presence of pathogen DNA in acute-phase blood by PCR, observation of morulae in circulating leukocytes on acute-phase blood smears, by culture, and/or by demonstration of a 4-fold increase in specific antibody (IgG + IgM) titer between acute- and convalescent-phase serum samples or a single acute-phase titer of ≥ 160 by indirect immunofluorescence assay (IFA) using *A. phagocytophilum*-infected HL-60 cells or *E. chaffeensis*-infected DH82 cells as antigens. The samples were blindly tested to reduce any possible bias during experimentation. Each DNA sample was tested three times and considered as positive if consistent in at least 2 out of 3 reactions. Details of diagnostic tests for each patient are shown in Table 2.

RESULTS

Identification of multicopy sequences in *A. phagocytophilum* genome and RPA assay design. Bioinformatics analysis of the *A. phagocytophilum* (HZ strain) complete genome sequence identified numerous repeated DNA fragments. One of these fragments is within *msp2* and has a total of 16 copies. A survey of eight other *A. phagocytophilum* strains with available whole-genome sequences revealed 12 to 21 copies with 100% sequence identity (Fig. 1A). A BLAST search of this DNA fragment with other species within the *Anaplasma* genus, including *Anaplasma marginale* and *Anaplasma centrale*, or other closely related species, such as *E. chaffeensis*, did not result in any significant homology. These indicate that this 171-bp region is well conserved within strains of *A. phagocytophilum* yet highly specific to *A. phagocytophilum* species, making it an ideal target for designing molecular detection assays. Three forward and three reverse RPA primers were designed and tested with conventional PCR for their performance (Fig. 1B and Table 1). Primers "F3" and "R3" were chosen due to high yield of amplicon, and a corresponding fluorescent probe was designed (Fig. 1C). To ensure the specificity of this primer set (F3/R3), Primer-BLAST was performed against available

TABLE 2 RPA assay using DNA extracted from clinical blood samples

Patient sample	<i>Anaplasma</i> detection by RPA ^a	<i>Anaplasma</i> detection by qPCR ^{a,b}	Clinical test results ^{a,c}			
			Serology	Culture	Blood smear	PCR ^d
01HE5	Neg	Neg	<i>E. chaffeensis</i>	<i>E. chaffeensis</i>	n.d.	<i>E. chaffeensis</i>
99HE26	Neg	Neg	<i>E. chaffeensis</i>	<i>E. chaffeensis</i>	Pos	<i>E. chaffeensis</i>
99HE9	Neg	Neg	acute only; negative	<i>E. chaffeensis</i>	Pos	<i>E. chaffeensis</i>
96HE19	Neg	Neg	<i>E. chaffeensis</i>	n.d.	n.d.	<i>E. chaffeensis</i>
14HE01	Neg	Neg	n.d.	Neg	Pos	<i>E. chaffeensis</i>
93HE4	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	Pos	<i>A. phagocytophilum</i>
93HE8b	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	Pos	<i>A. phagocytophilum</i>
95HE2	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	Pos	<i>A. phagocytophilum</i>
95HE8	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	Pos	<i>A. phagocytophilum</i>
96HE55	Pos	Pos	<i>A. phagocytophilum</i>	<i>A. phagocytophilum</i>	Pos	<i>A. phagocytophilum</i>
96HE75	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	Pos	<i>A. phagocytophilum</i>
06HE3	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	Pos	<i>A. phagocytophilum</i>
08HE03	Pos	Pos	acute only; negative	n.d.	Pos	<i>A. phagocytophilum</i>
96HE164	Pos	Pos	<i>A. phagocytophilum</i>	Neg	Pos	<i>A. phagocytophilum</i>
96HE165	Pos	Pos	<i>A. phagocytophilum</i>	Neg	Pos	<i>A. phagocytophilum</i>
97HE56	Pos	Pos	<i>A. phagocytophilum</i>	Neg	Pos	<i>A. phagocytophilum</i>
97HE57	Pos	Pos	<i>A. phagocytophilum</i>	Neg	Pos	<i>A. phagocytophilum</i>
97HE97	Pos	Pos	<i>A. phagocytophilum</i>	<i>A. phagocytophilum</i>	Pos	<i>A. phagocytophilum</i>
98HE4	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	Pos	<i>A. phagocytophilum</i>
98HE24	Pos	Pos	n.d.	n.d.	n.d.	<i>A. phagocytophilum</i>
98HE28	Pos	Pos	Neg	n.d.	n.d.	<i>A. phagocytophilum</i>
97HE300	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	n.d.	<i>A. phagocytophilum</i>
E-PCR72	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	n.d.	<i>A. phagocytophilum</i>
98HE3	Pos	Pos	<i>A. phagocytophilum</i>	Neg	Pos	<i>A. phagocytophilum</i>
E-PCR51	Pos	Pos	acute only; negative	n.d.	n.d.	<i>A. phagocytophilum</i>
96HE76	Pos	Pos	<i>A. phagocytophilum</i>	<i>A. phagocytophilum</i>	Pos	<i>A. phagocytophilum</i>
96HE73	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	Pos	<i>A. phagocytophilum</i>
96HE74	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	Pos	<i>A. phagocytophilum</i>
97HE242	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	n.d.	<i>A. phagocytophilum</i>
96HE68	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	n.d.	<i>A. phagocytophilum</i>
96HE53	Pos	Pos	negative	<i>A. phagocytophilum</i>	Pos	<i>A. phagocytophilum</i>
96HE77	Pos	Pos	<i>A. phagocytophilum</i>	<i>A. phagocytophilum</i>	Pos	<i>A. phagocytophilum</i>
96HE57	Pos	Pos	<i>A. phagocytophilum</i>	n.d.	n.d.	<i>A. phagocytophilum</i>
E-PCR91	Pos	Pos	acute only; negative	n.d.	n.d.	<i>A. phagocytophilum</i>
11HE09	Pos	Neg	acute only; negative	n.d.	n.d.	<i>A. phagocytophilum</i>
10HE08	Pos	Pos	acute only; negative	n.d.	n.d.	<i>A. phagocytophilum</i>
Normal human blood 2	Neg	Neg	n.d.	n.d.	n.d.	n.d.
Normal human blood 11	Neg	Neg	n.d.	n.d.	n.d.	n.d.
Normal human blood A	Neg	Neg	n.d.	n.d.	n.d.	n.d.
Normal human blood B	Neg	Neg	n.d.	n.d.	n.d.	n.d.
Normal human blood C	Neg	Neg	n.d.	n.d.	n.d.	n.d.
Normal	Neg	Neg	n.d.	n.d.	n.d.	n.d.

^aPos, positive; Neg, negative; n.d., not determined.^bUsing primer sets of both msp2F/msp2R and ApMSP2f/ApMSP2r in Table 1 for *A. phagocytophilum* detection.^cRefer to Materials and Methods for details on diagnosis.^dClinical test PCR, targeting either 16S rRNA or *msp2* genes, was performed at admitting hospitals using blood samples collected during acute phase of infection.

genomes of bacterial, viral, and tick species and human genome as well. No nonspecific amplification was detected.

Analytical limit of detection of the RPA assay. To evaluate the performance of the RPA amplification, we set out to determine its analytical sensitivity using serial dilutions of plasmid DNA containing the 171-bp target or *A. phagocytophilum* whole genomic DNA. We first generated a reference plasmid by inserting a DNA fragment covering the RPA amplicon region. Five to 1,000 copies of this plasmid in a 10- μ l volume were made by serial dilutions. Amplification was detected in all samples containing plasmids, and our RPA assay reliably detected the presence of 5 copies of plasmid within 10 min of reaction (Fig. 2A). Since the *A. phagocytophilum* Webster strain contains 19 copies of the 171-bp DNA fragment, we expect that, in theory, the RPA assay would be sensitive enough to detect even less than 1 GE copy of *A. phagocytophilum*. Indeed, when various GE copy numbers of *A. phagocytophilum* were used as template for RPA assay,

FIG 1 Design and evaluation of RPA primers and probe for a conserved multicopy DNA fragment in the *A. phagocytophilum* genome. (A) Bioinformatics analysis based on the whole-genome sequence of *A. phagocytophilum* HZ strain identified a well-conserved multicopy DNA fragment located within *msp2* (12 to 21 copies were found in various strains). (B) Three primers in either the forward or reverse direction were designed, and conventional PCR was performed to amplify *Anaplasma* genomic DNA using different combinations of primer sets as indicated. PCR products were analyzed by agarose gel electrophoresis. (C) Schematic illustration of the locations of primers and fluorescent exo probe for the RPA assay used in this study (FAM, carboxyfluorescein; THF, tetrahydrofuran; BHQ-1, Black Hole Quencher 1).

specific amplification was observed in reactions containing 1,000 to as little as 1 GE copy of *A. phagocytophilum* DNA (Fig. 2B). In order to test the analytical sensitivity of our RPA assay in human blood samples, we mimicked clinical patient samples by spiking DNA of various GE copies of *A. phagocytophilum* into 200 μ l of normal human blood. DNA was extracted and eluted into a 20- μ l elution buffer, 4 μ l of which was used for each real-time PCR or RPA reaction mixture. As demonstrated in Fig. 2C and D, while 5 GE copies of *A. phagocytophilum* DNA in 200 μ l blood can be detected in 2 out of 7 RPA reactions, 25 copies in 200 μ l whole-blood sample resulted in 100% detection rate (4 out of 4 reactions). Overall, the performance of our RPA assay was very similar to that of the real-time PCR assay targeting the same 171-bp region in terms of their limit of detection in mimicked clinical samples. These results indicate that the *A. phagocytophilum* RPA assay could be as sensitive as real-time PCR and offers reliable detection of this pathogen in human patients with 125 bacteria/ml in whole blood.

***A. phagocytophilum* RPA assay has high analytical specificity.** BLAST analysis indicated that the 171-bp DNA sequence did not share significant homology with any other species even within the *Anaplasma* genus; thus, we set out to confirm this by performing the RPA assay on DNA from a variety of sources, especially phylogenetically closely related species. As indicated in Fig. 3A and B, no amplification was observed when the following DNA templates were added: *Anaplasma platys* (1×10^4 copies), *E. chaffeensis* (Liberty strain, 1×10^4 copies), *B. burgdorferi* (B31 strain, 1×10^5 copies), *Orientia tsutsugamushi* (Karp strain, 2×10^4 copies), *Rickettsia rickettsii* (2×10^5 copies), *Rickettsia bellii* (2×10^5 copies), *Rickettsia prowazekii* (2×10^5 copies), *Rickettsia conorii* (2×10^5 copies), and human DNA (1×10^5 copies). These results indicate that the *A. phagocytophilum* RPA assay is highly specific and does not cross-react with human DNA or bacterial DNA from many closely related pathogenic species.

***A. phagocytophilum* RPA assay has high clinical sensitivity.** We next attempted to evaluate the clinical applicability of *A. phagocytophilum* RPA assay on DNA extracted from blood samples of 42 human patients or healthy blood donors (Fig. 4 and Table 2). As summarized in Table 2, the *A. phagocytophilum* RPA assay was able to identify 100%

FIG 2 Analytical limit of detection for the *A. phagocytophilum* RPA assay. (A) Plasmid containing RPA target sequence was serially diluted (1,000 to 5 copies) and used as template for RPA reaction mixtures. Fluorescent signals were monitored in real time in a Twista tube scanner. (B) *A. phagocytophilum* (Webster strain) DNA of 1 to 1,000 GE copies were used as template for amplification by RPA. (C) DNA was extracted from 200 μL of normal human whole blood spiked with 0 to 250 GE copies of *A. phagocytophilum* DNA and eluted into 20 μL elution buffer. Four microliters of eluted DNA was used as template for *A. phagocytophilum* RPA reaction mixtures. Summary of detection results using either real-time PCR (primer set msp2F/msp2R; Table 1) or RPA is shown (*, number of positive detections out of total number of reactions performed). (D) Representative real-time fluorescent signals from RPA reactions using expected GE copies per reaction as in panel C.

(31/31) of the patients that were diagnosed with HGA by serology, culture, blood smear, and/or PCR. Ehrlichiosis is caused by a very closely related bacterium, *E. chaffeensis*, and shares similar clinical symptoms of HGA. Serologic responses could be cross-reactive which confounds diagnosis. Among the five patients diagnosed with

FIG 3 High analytical specificity of *A. phagocytophilum* RPA assay. (A) Genomic DNA from various organisms, including *A. phagocytophilum* (Webster strain, 5 GE copies), *E. chaffeensis* (Liberty strain, 1×10^4 copies), *B. burgdorferi* (B31 strain, 1×10^5 copies), *Orientia tsutsugamushi* (Karp strain, 2×10^4 copies), *Rickettsia rickettsii* (2×10^5 copies), and human (1×10^5 copies), was used as template for RPA reaction mixtures. (B) Summary of RPA results using DNA from various organisms (at least 1×10^4 GE copies from each organism were used except for *A. phagocytophilum* at 250 copies).

FIG 4 High clinical sensitivity of *A. phagocytophilum* RPA assay. Representative real-time fluorescent signals from RPA reactions using 2 μ l of DNA extracted from human patient blood samples (see also Table 2). Signals from an *E. chaffeensis* infection patient (99HE9) sample overlapped with normal human blood at the bottom of the graph. Experiments were repeated at least three times for each DNA sample.

ehrlichiosis (all PCR positive), all tested negative by RPA assay as did six samples from healthy human blood. These data prove that our RPA assay is highly sensitive and specific for detecting *A. phagocytophilum* in clinical samples.

DISCUSSION

The incidence of HGA has increased dramatically during the past 20 years, and seroprevalences of 8.9% to 36% have been reported in certain parts of the United States (16, 17). Although the case fatality rate is low at 0.6%, 36% of patients develop disease symptoms that are severe enough to require hospitalization (18). Compared with traditional diagnostic methods, such as blood smear microscopy, serology, and culture, direct pathogen DNA detection offers sensitive and rapid diagnosis during the early acute phase of the infection, which is critical for effective antibiotic treatment. However, PCR-based assays (19) require expensive equipment, such as thermal cyclers and trained operators, which are not available in rural areas where the infection is more likely to occur. Sending patient samples to reference laboratories equipped with these resources will inevitably delay diagnosis and effective treatment. Thus, simple, rapid, and low-cost methods are in urgent need in these areas. In the present study, we developed a sensitive RPA assay by targeting a multicopy DNA fragment that is able to detect one GE copy of *A. phagocytophilum* within 10 min of reaction. The reagent costs for this assay are in the \$4 to \$5 range per sample, and a heat block that maintains temperature at 39°C is sufficient to complete the reaction. A fluorescence tube reader is required for detecting fluorescence released after amplification. However, this detection method could be substituted with a lateral flow strip test without reducing assay sensitivity (11). In addition, RPA assays have been found to be highly reproducible, at a comparable level to qPCR assays (20). In the present study, replicate tests of RPA assays on clinical/nonclinical samples have been performed on different days and by different individuals, and the results were highly reproducible. With further development, this assay could be a valuable tool for patient diagnosis and also for vector surveillance and epidemiologic studies in remote areas where resources are very limited.

Isothermal amplification for *A. phagocytophilum* was developed by Pan et al. using loop-mediated isothermal amplification (LAMP) (21). Compared with LAMP (22), the RPA assay is carried out at lower temperatures (37 to 42°C versus 60 to 65°C) with less reaction time (20 min versus 60 min). The limit of detection for the LAMP assay reported by Pan et al. is 25 copies per reaction mixture using reference plasmids, while our RPA assay is 5 copies. Furthermore, although the same *msp2* was used for both assays, the region for primer design used by Pan et al. has fewer copies compared with the 171-bp sequence of our RPA assay in genomes from both Webster and HZ strains. As shown in Fig. 1A, copy numbers of the 171-bp target region varies from 12 to 21 among nine *A. phagocytophilum* strains. In the present study, the Webster strain, which contains 19

copies of this fragment, was used for testing limit of detection (Fig. 2B to D). Thus, it is possible that our RPA assay will have slightly lower (e.g., Norway variant 2 with 12 copies) or higher (ApMUC09 with 21 copies) analytical sensitivities in detecting other strains of *A. phagocytophilum*. While this manuscript was in review, Zhao et al., published data on an RPA assay targeting *A. phagocytophilum* 16S rRNA gene (23). Since this is a single-copy gene, however, the analytical sensitivity of this assay is much lower (~22 GE copies) compared to ours (1 GE copy). The high analytical sensitivity of our RPA assay is expected to provide high sensitivity in clinical samples as well. Indeed, the RPA assay demonstrated 100% sensitivity to detect previously diagnosed clinical cases of *A. phagocytophilum* infection (31/31; Table 2). Consistent results on *Anaplasma* detection were obtained in all but one patient sample (11HE09) between the RPA and two qPCR assays performed in our laboratory (Table 2). When this patient (11HE09) was admitted at acute phase, the serology was negative; however, using qPCR, *A. phagocytophilum* DNA was detected in duplicate tests. Possible explanations for the negative qPCR results on this sample in the current study include DNA degradation with prolonged storage and/or target DNA at low enough levels that detection became stochastic. Surprisingly, DNA from 11HE09 consistently tested positive in the RPA assay. To rule out the possibility of a false-positive result, amplicons from the RPA reaction of 11HE09 DNA were purified and sequenced and confirmed to match the expected sequence within the 171-bp region. In terms of specificity, we evaluated the RPA reactions using DNA from a wide range of organisms, including human and phylogenetically closely related bacteria. No cross-reactivity was observed (Fig. 3A and B). We further tested our assay in multiple clinical cases of *E. chaffeensis* infection with positive molecular detection, and no amplification was detected. Taken together, we demonstrated high sensitivity and specificity for potential clinical applications of our RPA assay. However, given the high sensitivity of our assay to detect even one GE copy of *A. phagocytophilum* DNA, it is imperative to use caution and follow strategies to prevent nucleic acid contamination derived from DNA templates or amplicons. Some of the important measures that we have taken throughout this study included separating pre- and postamplification areas with dedicated equipment and supplies, routine cleaning of these areas with 0.5% sodium hypochlorite (10% bleach), and immediately discarding reaction tubes with amplicons in sealed plastic bags. We also prepared master mix for multiple reactions and always included no-template negative controls for each batch of RPA reaction mixtures.

In summary, a highly-conserved multicopy genomic region for *A. phagocytophilum* was identified upon which an isothermal RPA assay was designed and evaluated. This assay has an analytical limit of detection of one GE copy of *A. phagocytophilum* DNA and displayed 100% sensitivity and specificity in a set of well-defined clinical samples. This assay has a clear potential to be further developed into a point-of-care diagnostic or vector surveillance tools, which will be especially valuable in remote areas where resources are limited.

ACKNOWLEDGMENTS

We thank Emily Clemens, Angela Caranci, Zhiwen Zhang, and Tatyana Belinskaya for their technical assistance, Johan Bakken for identification and enrollment of patients with human granulocytic anaplasmosis, and the various physicians and health care practitioners who provided clinical samples that were used in this study. We are deeply indebted to the late Wei-Mei Ching who conceived and guided this study as a dedicated project leader.

This work was supported by work unit number 6000.RAD1.J.A0310 with funding from the Military Infectious Diseases Research Program (MIDRP) to W.-M.C.

C.-C.C. and W.-M.C. are U.S. Government employees, and the work of these individuals was prepared as part of official government duties. Title 17 U.S.C. §105 provides that "copyright protection under this title is not available for any work of the United States Government." Title 17 U.S.C. §101 defines a U.S. Government work as a work

prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

The views expressed are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of the Army, Department of Defense, or the U.S. Government.

W.-M.C. and C.-C.C. conceived the study. P.C. performed bioinformatics sequence analysis. L.J. performed the experiments. C.-C.C., L.J., J.S.D., and W.-M.C. analyzed and interpreted data. L.J. wrote the manuscript with contributions from all authors.

The authors declare no conflict of interest.

REFERENCES

1. Stuen S, Granquist EG, Silaghi C. 2013. *Anaplasma phagocytophilum*—a widespread multi-host pathogen with highly adaptive strategies. *Front Cell Infect Microbiol* 3:31. <https://doi.org/10.3389/fcimb.2013.00031>.
2. Bakken JS, Dumler JS. 2015. Human granulocytic anaplasmosis. *Infect Dis Clin North Am* 29:341–355. <https://doi.org/10.1016/j.idc.2015.02.007>.
3. Rand JV, Tarasen AJ, Kumar J, Homan SM, Tobin E. 2014. Intracytoplasmic granulocytic morulae counts on confirmed cases of ehrlichiosis/anaplasmosis in the northeast. *Am J Clin Pathol* 141:683–686. <https://doi.org/10.1309/AJCP6Q2BOKYALDYZ>.
4. Massung RF, Slater K, Owens JH, Nicholson WL, Mather TN, Solberg VB, Olson JG. 1998. Nested PCR assay for detection of granulocytic ehrlichiae. *J Clin Microbiol* 36:1090–1095. <https://doi.org/10.1128/JCM.36.4.1090-1095.1998>.
5. Courtney JW, Kostelnik LM, Zeidner NS, Massung RF. 2004. Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. *J Clin Microbiol* 42:3164–3168. <https://doi.org/10.1128/JCM.42.7.3164-3168.2004>.
6. de la Fuente J, Massung RF, Wong SJ, Chu FK, Lutz H, Meli M, von Loewenich FD, Grzeszczuk A, Torina A, Caracappa S, Mangold AJ, Naranjo V, Stuen S, Kocan KM. 2005. Sequence analysis of the *msp4* gene of *Anaplasma phagocytophilum* strains. *J Clin Microbiol* 43:1309–1317. <https://doi.org/10.1128/JCM.43.3.1309-1317.2005>.
7. Zanolli LM, Spoto G. 2013. Isothermal amplification methods for the detection of nucleic acids in microfluidic devices. *Biosensors (Basel)* 3:18–43. <https://doi.org/10.3390/bios3010018>.
8. Piepenburg O, Williams CH, Stemple DL, Armes NA. 2006. DNA detection using recombination proteins. *PLoS Biol* 4:e204. <https://doi.org/10.1371/journal.pbio.0040204>.
9. Kersting S, Rausch V, Bier FF, von Nickisch-Rosenegk M. 2014. Multiplex isothermal solid-phase recombinase polymerase amplification for the specific and fast DNA-based detection of three bacterial pathogens. *Mikrochim Acta* 181:1715–1723. <https://doi.org/10.1007/s00604-014-1198-5>.
10. Liu HB, Zang YX, Du XJ, Li P, Wang S. 2017. Development of an isothermal amplification-based assay for the rapid visual detection of *Salmonella* bacteria. *J Dairy Sci* 100:7016–7025. <https://doi.org/10.3168/jds.2017-12566>.
11. Chao CC, Belinskaya T, Zhang Z, Ching WM. 2015. Development of recombinase polymerase amplification assays for detection of *Orientia tsutsugamushi* or *Rickettsia typhi*. *PLoS Negl Trop Dis* 9:e0003884. <https://doi.org/10.1371/journal.pntd.0003884>.
12. Abd El Wahed A, El-Deeb A, El-Tholoth M, Abd El Kader H, Ahmed A, Hassan S, Hoffmann B, Haas B, Shalaby MA, Hufert FT, Weidmann M. 2013. A portable reverse transcription recombinase polymerase amplification assay for rapid detection of foot-and-mouth disease virus. *PLoS One* 8:e71642. <https://doi.org/10.1371/journal.pone.0071642>.
13. Amer HM, Abd El Wahed A, Shalaby MA, Almajhdi FN, Hufert FT, Weidmann M. 2013. A new approach for diagnosis of bovine coronavirus using a reverse transcription recombinase polymerase amplification assay. *J Virol Methods* 193:337–340. <https://doi.org/10.1016/j.jviromet.2013.06.027>.
14. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3—new capabilities and interfaces. *Nucleic Acids Res* 40:e115. <https://doi.org/10.1093/nar/gks596>.
15. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134. <https://doi.org/10.1186/1471-2105-13-134>.
16. Aguero-Rosenfeld ME, Donnarumma L, Zentmaier L, Jacob J, Frey M, Noto R, Carbonaro CA, Wormser GP. 2002. Seroprevalence of antibodies that react with *Anaplasma phagocytophilum*, the agent of human granulocytic ehrlichiosis, in different populations in Westchester County, New York. *J Clin Microbiol* 40:2612–2615. <https://doi.org/10.1128/jcm.40.7.2612-2615.2002>.
17. Bakken JS, Goellner P, Van Etten M, Boyle DZ, Swonger OL, Mattson S, Krueth J, Tilden RL, Asanovich K, Walls J, Dumler JS. 1998. Seroprevalence of human granulocytic ehrlichiosis among permanent residents of northwestern Wisconsin. *Clin Infect Dis* 27:1491–1496. <https://doi.org/10.1086/515048>.
18. Dahlgren FS, Mandel EJ, Krebs JW, Massung RF, McQuiston JH. 2011. Increasing incidence of *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* in the United States, 2000–2007. *Am J Trop Med Hyg* 85:124–131. <https://doi.org/10.4269/ajtmh.2011.10-0613>.
19. Silaghi C, Santos AS, Gomes J, Christova I, Matei IA, Walder G, Domingos A, Bell-Sakyi L, Sprong H, von Loewenich FD, Oteo JA, de la Fuente J, Dumler JS. 2017. Guidelines for the direct detection of *Anaplasma* spp. in diagnosis and epidemiological studies. *Vector Borne Zoonotic Dis* 17:12–22. <https://doi.org/10.1089/vbz.2016.1960>.
20. Shahin K, Gustavo Ramirez-Paredes J, Harold G, Lopez-Jimena B, Adams A, Weidmann M. 2018. Development of a recombinase polymerase amplification assay for rapid detection of *Francisella noatunensis* subsp. *orientalis*. *PLoS One* 13:e0192979. <https://doi.org/10.1371/journal.pone.0192979>.
21. Pan L, Zhang L, Wang G, Liu Q, Yu Y, Wang S, Yu H, He J. 2011. Rapid, simple, and sensitive detection of *Anaplasma phagocytophilum* by loop-mediated isothermal amplification of the *msp2* gene. *J Clin Microbiol* 49:4117–4120. <https://doi.org/10.1128/JCM.01085-11>.
22. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28:E63. <https://doi.org/10.1093/nar/28.12.e63>.
23. Zhao S, Cui Y, Jing J, Yan Y, Peng Y, Shi K, Wang K, Zhou Y, Jian F, Zhang L, Wang R, Ning C. 2019. Rapid and sensitive detection of *Anaplasma phagocytophilum* using a newly developed recombinase polymerase amplification assay. *Exp Parasitol* 201:21–25. <https://doi.org/10.1016/j.exppara.2019.04.010>.
24. Dong T, Qu Z, Zhang L. 2013. Detection of *A. phagocytophilum* and *E. chaffeensis* in patient and mouse blood and ticks by a duplex real-time PCR assay. *PLoS One* 8:e74796. <https://doi.org/10.1371/journal.pone.0074796>.