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Recombinase Polymerase Amplification Assay for Rapid Detection of *Francisella tularensis*

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Several real-time PCR approaches to develop field detection for *Francisella tularensis*, the infectious agent causing tularemia, have been explored. We report the development of a novel qualitative real-time isothermal recombinase polymerase amplification (RPA) assay for use on a small ESEQuant Tube Scanner device. The analytical sensitivity and specificity were tested using a plasmid standard and DNA extracts from infected rabbit tissues. The assay showed a performance comparable to real-time PCR but reduced the assay time to 10 min. The rapid RPA method has great application potential for field use or point-of-care diagnostics.

Because of its extraordinary infectiousness, the zoonotic pathogen *Francisella tularensis* causing tularemia was in the past the subject of state-run biowarfare research programs and therefore is included on the CDC category A list of biothreat agents. It causes disease in a vast range of animals, with relevant disease transmission to humans by direct contact or via vectors such as deer flies, horse flies, mosquitoes, and hard ticks. Infection due to inhalation of aerosols can occur through contact with infected hares. There are three *F. tularensis* subspecies, *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, and *F. tularensis* subsp. *mediasiatica*, which can be found in several environments and geographical regions. The first two subspecies and *Francisella novicida* cause the bulk of human infections (7, 19). Infection by *Francisella hispaniensis* has also been described, and results of phylogenetic analysis suggest that *F. novicida* should be regrouped as a fourth subspecies of *F. tularensis* (3, 12).

In all scenarios dealing with intentional release of biothreat agents, timely diagnosis is regarded as essential to identifying and containing outbreaks of infectious disease (4). Many efforts have been made to reduce the assay time of PCR-based nucleic acid detection. In spite of engineering constraints regarding temperature cycling needed for the PCR assays, short protocols and miniaturized cyclers or chip platforms are being developed (5, 6, 18).

In recent years a variety of isothermal amplification methods have been developed which offer the possibility of developing even simpler point-of-care systems. One example is the ESEQuant Tube Scanner device (Qiagen Lake Constance GmbH, Stockach, Germany). This device contains a sophisticated fluorescence sensor which slides back and forth under a set of eight tubes, collecting fluorescence signals over time and allowing for real-time documentation of increasing fluorescence signals. A combined threshold and signal slope analysis is used for signal interpretation, which can be confirmed by second-derivative analysis (11; also ESEQuant Tube Scanner software [Qiagen]). The recombinase polymerase amplification (RPA) assay is an isothermal amplification method which can be combined with a sequence-specific fluorescent probe for real-time detection. In RPA the phage-derived recombinase UvsX, assisted by its cofactor UvsY, aggregates with oligonucleotide primers to scan for homologous sequences in a DNA template. Upon identifying the specific homologous sequence, strand invasion and consequent strand displacement amplification via Sau polymerase (*Staphylococcus aureus*) starting from opposite primers generate amplified double-stranded DNA (dsDNA) copies in a similar way as PCR. The real-time probe format used is a probe (TwistAmp exo Probe) with a longer upstream stretch (30 nucleotides [nt]) carrying the fluorophore at its 3′ end, which is connected via a tetrahydrofuran (THF) spacer (dTFAM-THF-dTQuencher; FAM is 6-carboxyfluorescein) to an adjacent downstream smaller oligonucleotide (15 nt) carrying a 5′ quencher. Upon binding to the complementary sequence, an exonuclease recognizes the double-strand hybridization complex and cuts the spacer, leading the smaller quencher probe to dissociate and thus allowing for real-time fluorescence development on the longer probe hybridized to the accumulating amplified copies (20). Here, we describe the development of a real-time RPA on an ESEQuant tube scanner device for the detection of *F. tularensis*.

MATERIALS AND METHODS

Preparation of *F. tularensis* genomic DNA and DNA from hare samples. *Francisella* strains (Table 1) were grown in chocolate agar supplemented with IsoVitaleX (Becton, Dickinson, Madrid, Spain) and L-cysteine (Sigma-Aldrich Quimica SA, Madrid, Spain). Plates were incubated for 48 h at 37°C in an atmosphere with 5% CO_2. Colonies were resuspended in 200 μl of phosphate-buffered saline (PBS), and genomic DNA was extracted and purified with a QIAamp DNA minikit (Izasa S.A., Barcelona, Spain), following the instructions of the manufacturer. DNA was quantified by spectrophotometry with a Nano-Drop ND-1000 spectrophotometer (Nucliber, Madrid, Spain). DNA from naturally infected hare tissues was prepared in a biosafety level 3 (BSL-3) laboratory as follows. Snippets of

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TABLE 1 Bacterial strains tested

Strain[a]	Species[b]	RPA detection
LVS†	*F. tularensis* subsp. *holarctica*	+
FT 7†	*F. tularensis* subsp. *holarctica*	+
FT 10†	*F. tularensis* subsp. *holarctica*	+
B38†	*F. tularensis* subsp. *tularensis*	+
DuBa-1‡	*F. tularensis* subsp. *tularensis*	+
FHSP†	*F. hispaniensis*	+
FX 1†	*F. novicida*	+
FX 2†	*F. novicida*	+
U112†	*F. novicida*	+
CCUG4992†	*F. philomiragia*	−
03-1501‡	*Y. pestis*	−
ATCC 55075	*Y. enterocolitica*	−
ATCC 9610	*Y. enterocolitica*	−
ATCC 29833	*Y. pseudotuberculosis*	−
3007†	*B. anthracis*	−
ATCC 12759	*B. licheniformis*	−
ATCC 6633	*B. subtilis*	−
DSM 2046	*B. thuringiensis*	−
ATCC 14581	*B. megaterium*	−
DSM 299	*B. mycoides*	−
ATCC 31325	*B. polymyxa*	−
ATCC 53522	*B. cereus*	−
DSM 9378	*B. cereus*	−
DSM 6127	*B. cereus*	−
DSM 31	*B. cereus*	−
DSM 345	*B. cereus*	−
DSM 487	*B. cereus*	−
DSM 609	*B. cereus*	−
DSM 626	*B. cereus*	−
DSM 4490	*B. cereus*	−
DSM 6791	*B. cereus*	−

[a] Genomic DNA of the strains was provided as follows: †, Laboratorio de Espiroquetas y Patógenos Especiales, Servicio de Bacteriología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; ‡, Institute for Microbiology of the German Armed Forces. All other strains were provided by the Institute of Bacterial Infections and Zoonoses of the Federal Research Center for Animal Health. DNA concentrations of the DNA preparations ranged from 114,47 ng/μl to 563,59 ng/μl.

[b] *Y. pestis*, *Yersinia pestis*; *Y. enterocolitica*, *Yersinia enterocolitica*; *Y. pseudotuberculosis*, *Yersinia pseudotuberculosis*; *B. anthracis*, *Bacillus anthracis*; *B. licheniformis*, *Bacillus licheniformis*; *B. subtilis*, *Bacillus subtilis*; *B. thuringiensis*, *Bacillus thuringiensis*; *B. megaterium*, *Bacillus megaterium*; *B. mycoides*, *Bacillus mycoides*; *B. polymyxa*, *Bacillus polymyxa*; *B. cereus*, *Bacillus cereus*.

TABLE 2 Primers for the plasmid standard and for RPA

Name[a]	Sequence (5′–3′)
FTUL UP	TTATCTTTATCAATCGCAGGTTTAGC
FTUL DP	GGTTGGTGCACATGGCTAAGT
FT RPA FP	CACAAGGAAGTGTAAGATTACAATGGCAGGCTCC
FT RPA RP	CGCTACAGAAGTTATTACCTTGCTTAACTGTTA
FT RPA P	GTGCCATGATACAAGCTTCCCAATTACTAAG (BHQ1-dT)(THF)(FAMdT)GCTGAGAAG AACGATA(phosphate)[b]

[a] FTUL UP/DP, standard fragment upstream primer/downstream primer; FT RPA FP/RP, RPA forward and reverse primers; FT RPA P, RPA exo probe.

[b] BHQ1-dT, thymidine nucleotide carrying Black Hole Quencher 1; THF, tetrahydrofuran spacer; FAM-dT, thymidine nucleotide carrying fluorescein; phosphate, 3′ phosphate to block elongation.

hare tissue were homogenized in a pestle and incubated at 56°C overnight in 180 μl of the kit's ALT buffer (QIAamp DNA Kit Mini) and 20 μl of protease. DNA was subsequently extracted using the tissue protocol of the QIAamp DNA blood kit (Qiagen, Hilden, Germany) as recommended by the manufacturer.

Plasmid DNA standard and real-time PCR. The *tul4* gene was amplified using the primers FTUL UP/DP (Table 2) and the genomic DNA of *F. tularensis* strain DuBa-1 using the following PCR protocol. The reaction mixture (50 μl) contained 30 ng of DNA, 200 nM each primer, 200 μM each deoxynucleoside triphosphate (dNTP), 1U of *Taq* polymerase, and 1× *Taq* buffer (5 Prime, Hamburg, Germany). The temperature profile consisted of activation at 95°C for 1 min followed by 30 cycles of PCR at 95°C for 1 min, 60°C for 1 min, and 68°C for 1 min. The PCR product was ligated into pCRII and transformed into One Shot INVαF′ chemically competent *E. coli* cells (Invitrogen, Darmstadt, Germany). The plasmid was prepared, sequenced, and quantified using a PicoGreen reagent kit (Invitrogen, Darmstadt, Germany) as previously described (29). The standard was tested using real-time PCR for the *tul4* gene as described previously (8). The rabbit samples were additionally tested with a second real-time PCR for the 23-kDa protein gene (16). The real-time PCR assays were performed on a LightCycler, version 2.0, using a FastStart TaqMan Probe Master Kit (Roche, Manheim, Germany) and the second-derivative method for analysis; the assays showed the same sensitivities as reported in the original publications.

Real-time RPA amplicon design. The RPA amplicon for the detection of the *tul4* gene of *F. tularensis* was designed using the following available GenBank sequences: M32059, EF208970 to EF208977, and EF208979. In reference to sequence M32059 (*tul4* from nt 551 to 1000), the RPA amplicon was placed between nt 792 and 936 (length, 144 nt). The RPA probe was synthesized by TIB Molbiol (Berlin, Germany) using an inverse arrangement of fluorophore and quencher (dTBHQ1-THF-dTFAM).

RPA conditions. RPA was performed in a 50-μl volume using a TwistAmp exo kit (TwistDX, Cambridge, United Kingdom), 420 nM each RPA primer, 120 nM FAM-tagged RPA probe, 14 mM Mg acetate, and 1× rehydration buffer. All reagents except for the template or sample DNA and Mg acetate were prepared in a master mix, which was distributed into each 0.2-ml reaction tube containing a dried enzyme pellet. Mg acetate was pipetted into the tube lids. Subsequently, 1 μl of standard DNA or genomic DNA or 10 μl of DNA eluate extracted from rabbit tissues was added to the tubes. The lids were closed, the Mg acetate was centrifuged into the tubes using a minispin centrifuge, and the tubes were immediately placed into a ESEQuant Tube Scanner device (Qiagen Lake Constance, Stockach, Germany). Fluorescence measurements (excitation, 470 nm; detection, 520 nm [FAM channel]) were performed at 42°C for 20 min. This reaction temperature was found to yield the best performance in terms of sensitivity in a range tested from 39°C to 42°C. The tube scanner software offers threshold validation, i.e., evaluation of fluorescence by increase of fluorescence above three standard deviations over the background determined in minute 1 (adaptable) of the reaction. Additionally, the slope of the curve as mV/time can be used (slope adaptable). A second-derivative window calculating the turning point of the upward fluorescence development helps to verify curves with a very low slope.

Determination of sensitivity and specificity. The *F. tularensis tul4* gene plasmid standard was tested in eight replicates, the threshold time (in minutes) was plotted against the number of molecules detected, and a semilogarithmic regression was calculated. For exact determination, a probit regression (23) was performed using the Statistica software (StatSoft, Hamburg, Germany). The final assay was tested with DNA from bacterial strains listed in Table 1.

RESULTS

RPA sensitivity. Using the plasmid standard, the real-time PCR showed a sensitivity of 10^2 molecules detected, whereas the RPA showed a sensitivity of 10^2 to 10^1 molecules detected (Fig. 1A and B). Using the results of eight complete molecular standard runs, a probit model of regression predicted that in 95% of cases the RPA detects 19.01 molecules (Fig. 1C). The RPA was tested with several

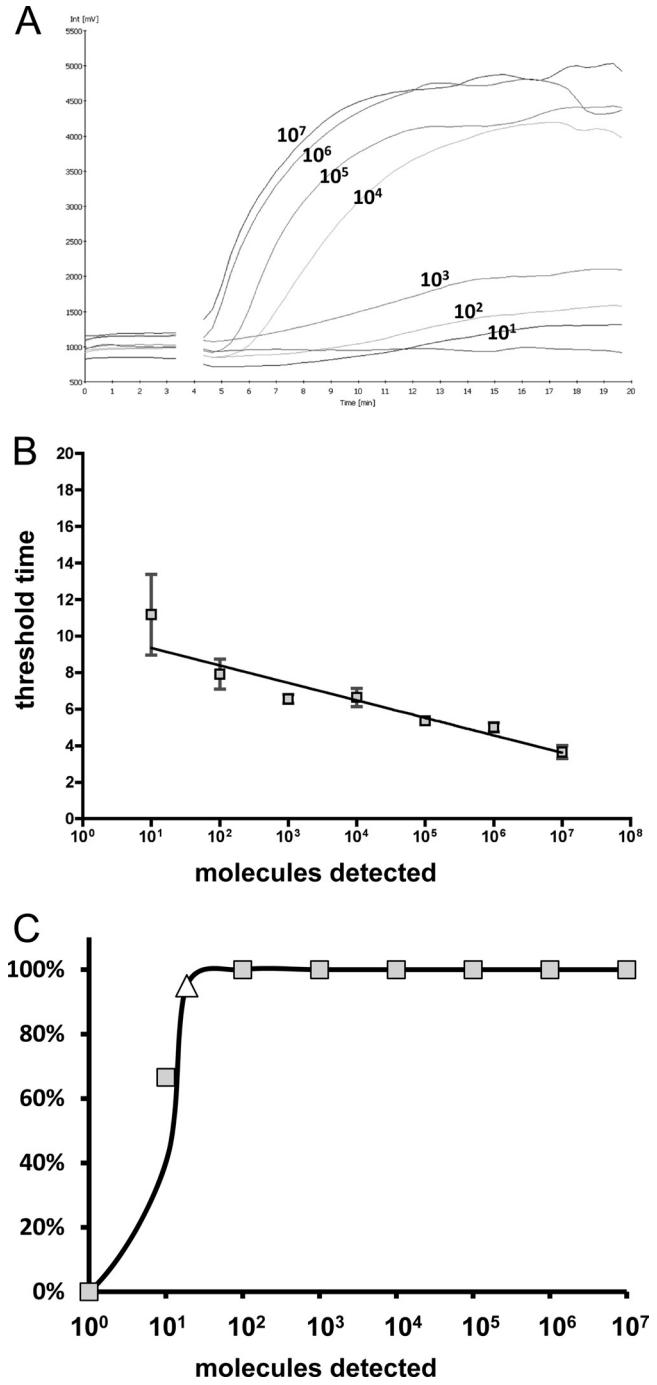

FIG 1 Real-time recombinase polymerase amplification assay performance. (A) Original graph of fluorescence development over time from the tube scanner software. (B) Analytical sensitivity as determined on a plasmid standard. Semilogarithmic regression of data from eight runs is shown. (C) Probit regression with the data of eight runs. Triangle, calculated detection limit in 95% of cases. Int., intensity of fluorescence.

F. tularensis strains (Table 1). It detected strains of *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *tularensis*, *F. hispaniensis*, and *F. novicida*, but it did not detect *F. philomiragia* (DNA concentration range, 114.47 ng/μl to 563.59 ng/μl).

Specificity. The RPA did not detect the genomic DNA of four *Yersinia* strains and 17 *Bacillus* strains. Nine hare tissue samples of naturally infected hares were tested for the presence of *F. tularensis* DNA. The DNA extracted from the samples was tested by two real-time PCRs and the new RPA. The RPA was almost as good as the *tul4* PCR (8) but detected fewer samples than the 23-kDa gene PCR (16) (Table 3).

DISCUSSION

There are several isothermal amplification methods that have been developed in the recent decade. Apart from T7 promoter-driven amplifications (transcription-mediated amplification [TMA], nucleic acid sequence-based amplification [NASBA], and single-primer isothermal amplification [SPIA]), there are strand displacement methods (strand displacement amplification [SDA] loop-mediated isothermal amplification [LAMP], and SmartAmp), helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA), and several rolling circle amplification (RCA) methods (1, 2, 10, 13, 25).

Most fluorescent formats for isothermal amplification use nonspecific intercalating fluorophores or fluorescent primers (LAMP, SDA, HDA, and RCA). For specific detection, probe formats have been developed for NASBA, RCA, HDA, and RCA (14, 17, 20, 26, 28). We chose to test the RPA method for several reasons: (i) the RPA method allows verification of an exponentially amplified product using a fluorescent probe, (ii) the RPA mixture contains the single-strand binding protein GP32, which has been shown to enhance nucleic acid detection (30), and (iii) the reagents are commercially available in a dried pellet format which makes the reaction amenable to field use or point-of-care applications.

In the past, several real-time PCR protocols for the detection of *F. tularensis* have been described targeting the *fopA*, 23-kDa, and *tul4* genes, the RD1 region, and the insertion element IS*Ftu*2 (24). The *tul4* gene detection assays appear to show the highest sensitivity. We established a published *tul4* gene-targeting real-time PCR (8) and tested it using a *tul4* gene plasmid standard created in our laboratory. It showed an analytical sensitivity of 10 molecules detected (data not shown). For this study, we designed an RPA for

TABLE 3 Rabbit tissues tested and test results

Sample no.[a]	Hare tissue	qPCR for the indicated gene[b]		RPA[d] for *tul4*
		23-kDa gene	*tul4*	
10TO174 B2	Bone marrow	30.13	29.07	7.7
10TO183 B10	Tongue	31.63	29.70	9.3
10TO179 B7	Spleen	34.12	32.66	10.0
10TO173 B1	Liver	36.12	34.77	11.7
10TO176 B4	Lymph node (gut)	38.46	37.25	Neg
10TO180 B8	Kidney	41.37	40.31	Neg
10TO178 B6	Muscle	41.35	Neg[c]	Neg
10TO175 B3	Lung	43.10	Neg	Neg
10TO182 A2	Brain	41.28	Neg	Neg
11T0351 (NI)	Spleen	Neg	Neg	Neg
11T0350 (NI)	Liver	Neg	Neg	Neg

[a] DNA concentration of the extracts ranged from 2.32 to 6.77 ng/μl. NI, not infected.
[b] qPCR, quantitative PCR. PCR results for the 23-kDa gene (16) and *tul4* (8) are given as C_T values analyzed with the fit points method on the LightCycler, version 2.0.
[c] Neg, negative.
[d] RPA values are given as threshold time values.

the same target region, tested it on the same plasmid standard, and achieved comparable analytical sensitivity (Fig. 1B), which we additionally analyzed by probit analysis (Fig. 1C). This clearly demonstrated that the isothermal real-time RPA has the same sensitivity as real-time PCR. The speed of the reaction, however, is much higher, yielding results in 10 min (Fig. 1A and B). Although in principle quantitative real-time RPA (qRPA) is possible, the TwistAmp exo kit currently has not yet been adapted for qRPA. The emphasis is on a robust reaction, which makes it an ideal tool for qualitative real-time RPA (20; also O. Piepenburg, personal communication).

Using several target genes simultaneously was shown to elevate the sensitivity of *F. tularensis* detection (27). We therefore additionally established a 23-kDa gene real-time PCR (16) and tested tissue samples of infected hares using both real-time PCRs and the new RPA. The 23-kDa gene real-time PCR detected *F. tularensis* in 9/9 rabbit tissue samples, the *tul4* gene real-time PCR found 6/9 samples as positive, and the *tul4* gene RPA found 4/9 samples positive. Thus, the 23-kDa gene real-time PCR appeared more sensitive than the *tul4* gene real-time PCR, and the RPA did not detect samples that had tested positive in the real-time PCR assays between threshold cycles C_T 35 and 40. In diagnostic real-time PCR assays, it is customary to regard results between C_T 35 and 40 as equivocal and above C_T 40 as negative. This particular RPA therefore is almost as sensitive as the real-time PCR, and the observed reduced sensitivity of the RPA assay misses the range of equivocal or false positives of the tested real-time PCRs.

Among the *F. tularensis* strains tested, all human pathogens were detected except for *F. philomiragia*, which in general is a fish pathogen and has been described to infect patients with an underlying chronic disease (9, 21, 22, 31). However, there may be more *Francisella* species or strains that potentially can infect humans, as a recent first report from the Southern Hemisphere indicates (http://www.promedmail.org/direct.php?id=20111105.3299).

This novel RPA therefore seems well suited for a biothreat first-line detection device able to detect pathogenic *F. tularensis* strains at high sensitivity.

The robust tube scanner device has a great potential to develop into an isothermal nucleic acid detection device for point-of-care detection and field use. It is much lighter and smaller than the R.A.P.I.D. or the RAZOR instrument for which real-time PCR assays for *F. tularensis* have been developed and evaluated (6, 16). RPAs have already been integrated onto a foil-based microfluidic LabDisc system (15). This could eventually lead to the development of RPA panels, e.g., for the simultaneous detection of category A infectious agents in small point-of-care devices.

In summary we have developed a very rapid and highly sensitive isothermal real-time RPA for the detection of *F. tularensis* on a mobile device. The results merit further investigation into this methodology.

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