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5′ Nuclease PCR Assay To Detect *Yersinia pestis*

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The 5′ nuclease PCR assay uses a fluorescently labeled oligonucleotide probe (TaqMan) to rapidly detect and quantitate DNA templates in clinical samples. We developed a 5′ nuclease PCR assay targeting the plasminogen activator gene (*pla*) of *Yersinia pestis*. The assay is species specific, with a detection threshold of 2.1×10^5 copies of the *pla* target or 1.6 pg of total cell DNA. The assay detected *Y. pestis* in experimentally infected *Xenopsylla cheopis* fleas and in experimentally infected monkey blood and oropharyngeal swabs. The TaqMan assay is simple to perform and rapid and shows promise as a future field-adaptable technique.

PCR techniques have significantly improved the detection and identification of bacterial pathogens. Because of their high specificity and sensitivity, PCR-based assays can be extremely important when rapid, accurate identification of pathogenic bacteria is required. Several PCR assays for the detection of *Yersinia pestis*, the causative organism of plague, have been developed (2, 7, 8, 15, 20). While the PCR assays offer high degrees of specificity and sensitivity, they are currently not adaptable to the processing of large numbers of samples. Therefore, we were interested in applying newer nucleic acid-based assays for detection of *Y. pestis*.

We employed the 5′ nuclease PCR assay (9), which exploits the ability of *Taq* polymerase to cleave nucleotides from an oligonucleotide probe annealed to a target DNA strand (6). The assay previously was used to detect *Listeria monocytogenes* (1), *Salmonella* spp. (4), orthopoxviruses (10), hepatitis C virus (14), and papillomaviruses (19) in a variety of clinical specimens.

In a positive assay, the probe will bind to its target site on the DNA strand downstream from the primer binding site. As the polymerase processes the newly synthesized leading strand, it encounters the bound probe and cleaves individual nucleotides from the 5′ end (eventually, the probe is degraded). The emission of the 6-carboxyfluorescein (FAM) moiety is normally quenched by virtue of its proximity to the dye 6-carboxytetramethylrhodamine; when the FAM moiety is cleaved from the oligonucleotide by the activity of the polymerase, its fluorescence is detected by a charge-coupled device camera. As the amplification reactions proceed, more amplicons become available for probe binding, and consequently, the fluorescence signal intensity per cycle increases. Because of the specificity of the probe, the possibility of false-positive reactions due to a fluorescence signal associated with the generation of nontarget amplicons does not exist with this system.

The increased signal intensity is detected and interpreted by the ABI 7700 Sequence Detector (Applied Biosystems, Foster City, Calif.), a combination thermal cycler, laser, and detection/software system. Once the amplification reaction is completed, analysis, and display of positive samples, can be done in less than 60 s. The ABI 7700 employs a computer algorithm to calculate a value termed ΔRn as follows: ΔRn = Rn+ − Rn−, where Rn+ is the emission intensity of the reporter divided by the emission intensity of the quencher at any given time in a reaction tube and Rn− is the emission intensity of the reporter divided by the emission intensity of the quencher measured before PCR amplification in that same reaction tube (6). Data for individual reactions are graphically displayed as ΔRn on the *y* axis, and cycle number is on the *x* axis. A threshold, defined as 10 standard deviations above the mean baseline fluorescence observed in no-template controls, from cycles 3 to 15, is also displayed on the graph. A reaction is considered positive if its ΔRn curve exceeds the threshold at the completion of 40 cycles. Alternatively, results can be qualitatively displayed in a 96-well format, with the positivity of each well indicated by gradations in color (white is most positive, and dark blue is less positive).

The ABI 7700 Sequence Detector allows quantitation of starting material in unknown samples, provided a series of standards are included in each assay. When the threshold cycle, Ct, for each standard is plotted on the *y* axis and the starting quantity for each standard is plotted on the *x* axis, a standard curve can be plotted. This standard curve can be used to quantify the template in unknowns. The Sequence Detection System 1.0 version software calculates and displays starting quantities for each sample when the data are presented in the 96-well format.

Here, we describe a TaqMan 5′ nuclease assay for rapid detection of *Y. pestis* in both nonhuman primate clinical specimens (from aerosol-infected monkeys) and experimentally infected vector fleas (*Xenopsylla cheopis*).

(Part of this work was previously presented as a poster at a Department of Defense meeting.)

MATERIALS AND METHODS

Bacterial species and strains. Table 1 lists the bacterial species and strains used. Some were obtained from the American Type Culture Collection, Rockville, Md., and some were from strains and stocks maintained at the U.S. Army Medical Research Institute of Infectious Diseases, while others were obtained from clinical isolates obtained at the Frederick Memorial Hospital in Frederick, Md., and identified by the Microscan protocol.

Total cell DNA was extracted from cultures of these bacteria with the Qiagen Tissue Kit (Qiagen Corp., Santa Clarita, Calif.), and 5 μl was used as a template. The person performing the assay did not know the identities of the samples.

To determine the detection limit of the assay, we used genomic (combined chromosomal and plasmid) DNA extracted from *Y. pestis* K25 cells as the template. Additionally, the 344-bp PCR product was cloned into *Escherichia coli*

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TABLE 1. Specificity of the *Y. pestis* TaqMan 5′ nuclease PCR assay

Species	Isolate	Ct[a]
Aeromonas hydrophila	M10915#2	31.7
Bacillus anthracis	Ames	30.6
B. anthracis	Colorado	30.6
B. anthracis	Vollum	40.0
Coxiella burnetii	Ohio	40.0
Enterobacter aerogenes	M8001	33.6
E. cloacae	ATCC 49141	32.0
E. faecalis	ATCC 49149	32.8
E. sakazakii	M12662	40.0
Escherichia coli	M127495	40.0
Haemophilus influenzae	ATCC 10211	40.0
Klebsiella oxytoca	ATCC 49131	31.7
K. pneumoniae	ATCC 49472	35.4
Normal throat flora		40.0
Normal fecal flora		40.0
No-template control		40.0
Proteus aeruginosa	ATCC 27853	40.0
P. mirabilis	M12702-1	40.0
Salmonella sp. (group B)	M14666	40.0
Shigella sonnei	M11098	37.4
Staphylococcus aureus	ATCC 29247	33.7
S. agalactiae	M10828	31.6
S. epidermidis	ATCC 49134	33.6
Serratia marcescens	M10759	40.0
Streptococcus pyogenes	M10780	40.0
Vibrio cholerae	N16961-K004	31.7
Yersinia enterocolitica	Hobs	40.0
Y. enterocolitica	E268	40.0
Y. enterocolitica	ATCC 9610	40.0
Y. pestis	195/P	15.5
Y. pestis	A1122	22.0
Y. pestis	Angola	18.3
Y. pestis	CO92	18.4
Y. pestis	Java-9	18.2
Y. pestis	K25	14.7
Y. pestis	Nairobi	15.0
Y. pestis	Pestoides A	18.6
Y. pestis	Pestoides Ba	17.6
Y. pestis	Pestoides C	18.4
Y. pestis	Pestoides D	22.2
Y. pestis	Pestoides E	37.6
Y. pseudotuberculosis	ATCC 6902	34.4
Y. pseudotuberculosis	ATCC 6903	32.7
Y. pseudotuberculosis	ATCC 6904	34.5

[a] Ct, threshold cycle value as calculated by SDS 1.0.5d30 software. Values of <25 are considered to be positive.

(T/A Cloning System; Invitrogen, San Diego, Calif.) and used as a template. The size of the target was 4.23 kb (vector plus insert); this is equivalent to 2.8×10^6 Da. Multiplying this value by the conversion factor 1.650×10^{-24} gives 4.70×10^{-18} g. One nanogram (10^{-9} g) therefore contained 2.1×10^8 copies, and 1 pg contained 2.1×10^5 copies. Converting these values to numbers of individual bacteria was problematic because we did not know the copy number of the 9.7-kb plasmid in our *Y. pestis* strains. Based on the estimation of 4,398 kb of DNA (chromosomal DNA and that of three different plasmids) per wild-type *Y. pestis* genome provided by Lucier and Brubaker (13), if we assume one copy per cell, the lower detection limit was theoretically 333 genomes/1.6 pg of total cell DNA; 10 copies per cell, 3,330 genomes; etc.

The more imaginative reader may equate copy number with CFU; we refrain from stating results in terms of this parameter because 1 CFU of *Y. pestis* may not always be equivalent to one viable bacterium, particularly in clinical samples.

Infected fleas. *X. cheopis* fleas originated from a colony maintained at Rocky Mountain Laboratories, Hamilton, Mont. Fleas were fed blood containing 5×10^8 CFU of *Y. pestis* KIM6 per ml by using an artificial feeding device (8). This strain of *Y. pestis* lacks the 70-kb virulence plasmid but does carry the 9.7-kb plasmid containing the plasminogen activator gene (*pla*) target of the TaqMan 5′ nuclease assay. Control group fleas received uninfected blood. Within 1 h after feeding, engorged fleas were frozen at −70°C. After shipment to the U.S. Army Medical Research Institute of Infectious Diseases, fleas were stored at 4°C and prepared for PCR by triturating individuals and batches of 5 and 10 fleas with plastic pestles (Kontes Instuments, Vineland, N.J.) in 1.5-ml microcentrifuge tubes. Tubes contained either 25 μl of Tris-EDTA (TE) buffer, phosphate-buffered saline or buffer AL (Qiagen Corp.). For some samples, 10 μl of crude homogenate was used as the template; for others, the homogenate was subjected to a DNA extraction procedure (see below). The DNA was resuspended in 10 μl of TE buffer, and 5 μl was used as a template.

Infected monkey tissues. Samples originated from African green monkeys (*Cercopithecus aethiops*) used in a study of experimental pneumonic plague (5). During that study, monkeys were exposed to *Y. pestis* CO92 by using nebulizer-generated aerosols containing a range of 1.4×10^2 to 9.2×10^4 CFU. At necropsy, tracheal fluid, blood, and serum were collected and cotton-tipped swabs were used to sample the oropharyngeal cavities. Material was gamma irradiated and stored at −70°C. After thawing, approximately 1 ml of each sample was added to 400 to 500 μl of detergent buffer AL (Qiagen Corp.) and incubated at 65°C for 10 min to completely deactivate any infectious agents. The swabs were allowed to thaw in 500 μl of buffer AL at 55°C with vigorous twirling to dislodge the contents. The samples were subjected to treatment with proteinase K solution at 55°C for 1 h, followed by two rounds of phenol-chloroform-isoamyl alcohol (PCI) extraction (Sigma Chemical Co., St. Louis, Mo.). DNA was precipitated with 100% ethanol, washed with 70% ethanol, resuspended in 50 μl of TE buffer, and quantitated with a spectrophotometer. Ten microliters of DNA (equivalent to 0.8 to 3.0 μg) was used as a template.

As a negative control, a swab was obtained from the nasal cavity of one of the investigators and processed and assayed in parallel with the monkey samples.

Primers and probes. Primers Yp pla IS1 (5′ TGC TTT ATG ACG CAG AAA CAG G 3′) and Yp pla IA2 (5′ CTG TAG CTG TCC AAC TGA AAC G 3′) were used to amplify a 344-bp region spanning residues 425 to 769 of the *Y. pestis pla* gene (18). The TaqMan probe used was MSI002 (5′ FAM TCT CAT CCT GGA GTA ACC AGC CTT TCA TAMRA p3′), located at residues 507 to 533 (reverse complement). FAM is the reporter dye attached to the 5′ thymine residue, and TAMRA is the quencher dye 6-carboxytetramethylrhodamine attached to the 3′ nucleotide (phosphate is also attached to the 3′ thymine residue to prevent extension of the bound probe during amplification).

As a control for the quality of templates extracted from clinical samples, primers specific for the human β-globin gene were used; these were capable of amplifying a band(s) from primate samples. The sequence of the forward primer was 5′ GAA GAG CCA AGG ACA GGT AC 3′, and that of the reverse primer was 5′ CAA CTT CAT CCA CGT TCA CC 3′, with a product size of 250 bp. These primers were used at an annealing temperature of 50°C.

5′ nuclease PCR. The PCRs were performed in 50-μl volumes containing 10 pmol of probe, 0.5 to 1.0 μM each primer, 6.0 mM $MgCl_2$, 1 U of Amplitaq polymerase, 1 μl of a nucleotide mixture (200 μM each deoxynucleotide triphosphate), and 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl). Cycling conditions were 1 to 2 min at 95°C for initial denaturation, followed by 15 s at 95°C and 1 min at 60°C for 40 to 50 cycles. The amplifications were performed with an ABI 7700 Sequence Detector in optical tubes and caps. When the reaction was complete, data were analyzed by the SDS 1.0.5d30 (beta version) software installed on the Sequence Detector.

RESULTS

Detection limit and specificity. The assay reproducibly detected 8 pg of genomic DNA isolated from strain K25 cells (Fig. 1). The assay often (four out of five times) detected the 1.6-pg dilution (Fig. 1), but this was not as reproducible, as the curves for the no-template controls sometimes overlapped those of the standard. When a cloned insert was used as the template, the detection limit was 1 pg or 2.1×10^5 copies (data not shown).

The specificity of the assay was tested against a panel of bacterial templates representing 43 genera and species (Table 1). Only *Y. pestis* K25, CO92, 195/P, A1122, Nairobi, Java 9, Angola, and pestoides A, Ba, C, and D reacted with the probe. These samples gave threshold cycle (Ct) values of <25 cycles, which we designated as the upper limit of positivity. Strain pestoides E, which lacks the *pla* gene, and other *Yersinia* species (*Y. pseudotuberculosis* and *Y. enterocolitica*) were essentially nonreactive to the probe (Table 1), with Ct values of >25 cycles, confirming the species-specific nature of the assay.

Animal specimens. DNA was extracted from blood, tracheal fluid, serum, and oropharyngeal swabs and used as the template for the TaqMan assay. For monkey 5899AG, all samples (blood, swab, and tracheal fluid) were positive. For monkey T652, blood was negative and serum and swab samples were positive. For monkey 5296, blood and serum samples were

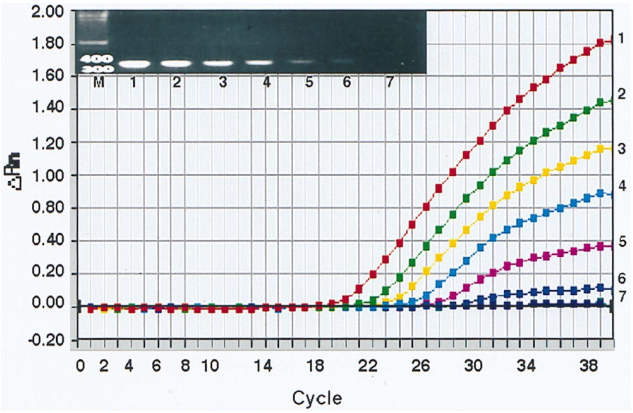

FIG. 1. Sensitivity of the TaqMan assay for *Y. pestis*. Total (chromosomal and plasmid) DNA isolated from *Y. pestis* K25 cells was used as the template in quantities of 5, 1, 0.2, 0.04, 0.008, and 0.0016 ng. Primers for a 344-bp region of the *Y. pestis pla* gene were used with 10 pmol of the MS002 probe per sample. The graph depicts amplification plots for each template; plot 1 is the 5-ng template, etc. Plot 7 is a no-template control. Inset: agarose gel electrophoresis image of respective PCR products. Lane M, 100-bp DNA ladder. Lane 1 corresponds to plot 1, etc. Lane 7 is a no-template control. (This figure was previously presented as a poster at a Department of Defense meeting.)

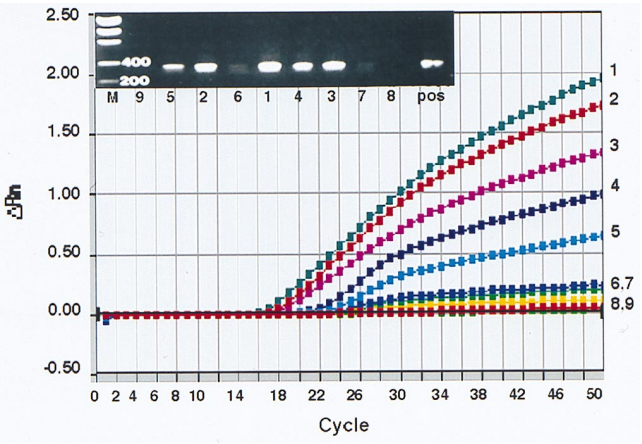

FIG. 3. Results of *Y. pestis* TaqMan assays performed on infected *X. cheopis* fleas with *Y. pestis pla* primers and 10 pmol of the MS002 probe per sample. The graph depicts the amplification plots for eight fleas (plots 1 to 8) and a no-template control (plot 9) (for clarity, positive control amplification plots have been omitted). The inset is an agarose gel electrophoresis image of the amplification products from fleas (samples 1 to 8) and a no-template control (sample 9). Lane M is a low-mass DNA ladder. Lane pos is a *Y. pestis* DNA positive control.

negative and the swab was positive. All swabs gave excellent ΔRn values (range, 1.7 to 2.0; Fig. 2) with Ct values ranging from 14.7 to 18.3, indicating that the starting quantity of the template in these samples was greater than that of the 5-ng total cell DNA standard (Fig. 2). The nasal swab obtained from one of the investigators and used as a negative control did not react with the probe (Fig. 2).

Infected fleas. When 10-μl aliquots of crude homogenate from individual fleas were used as templates, no amplification was observed. When DNA was extracted from this homogenate and used as the template, 2 of 15 fleas were positive by the TaqMan assay (data not shown). Subsequently, DNAs from eight individual fleas were obtained by using two PCI extraction steps. When this DNA was used as the template, five of the eight fleas were positive (Fig. 3). Of these five fleas, three had Ct values (17.3, 16.0, and 16.9) indicating that they contained more *Y. pestis* DNA than the 5-ng standard (Ct = 18.2). Two other fleas had Ct values (20.5 and 22.0) equivalent to the 1.0- and 0.2-ng standards, respectively.

As Fig. 3 indicates, two of the eight fleas were not positive by TaqMan assay but did have faint PCR products made visible by using a high level of enhancement in the gel imaging device (Eagle Eye II; Stratagene, La Jolla, Calif.). Bands were not visible with a conventional UV transilluminator. As controls, eight fleas fed uninfected blood were assayed and found to have no reactivity to the probe, and no PCR products were visible on agarose gels (data not shown).

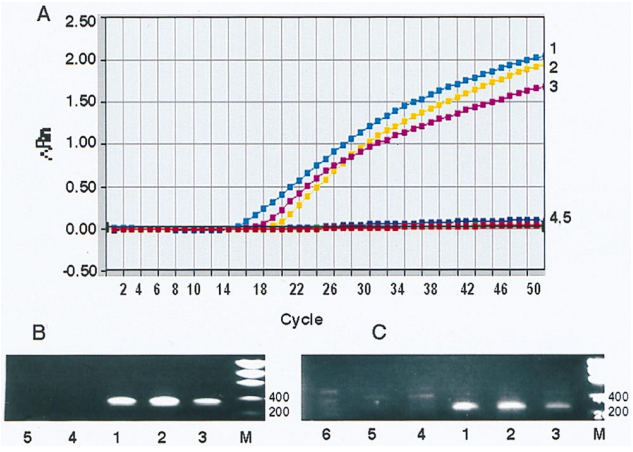

FIG. 2. Results of TaqMan assays performed on oropharyngeal swabs from monkeys experimentally aerosol infected with *Y. pestis*. The assay used *pla* primers and 10 pmol of the MS002 probe per sample. (A) Graph depicting the amplification plots for three monkeys (plots 1 to 3) and two no-template controls (plots 4 and 5; for clarity, positive control amplification plots have been omitted). (B) Agarose gel electrophoresis image of *pla* amplification products. Lanes: 4 and 5, no-template and extraction controls, respectively; 1 to 3, monkey samples; M, low-mass DNA ladder. (C) Agarose gel electrophoresis image of β-globin primer amplification products. Lanes: 6, human DNA positive control; 5, contamination control; 4, extraction control; 1 to 3, monkey samples; M, low-mass DNA ladder.

DISCUSSION

Our TaqMan 5′ nuclease PCR assay for detection of *Y. pestis* exhibited satisfactory levels of sensitivity (in the picogram range for both purified bacterial genomic DNA and cloned target sequences) and specificity (it was species specific). Results were obtained in 2 to 3 h when the ABI 7700 Sequence Detector was used, obviating the need for agarose gels, UV light, ethidium bromide, etc. The sequence detector allows quantitation of samples when a series of standards is included in the assay. Such features are important when rapid diagnosis is vital.

Other PCR assays for *Y. pestis* have reported greater sensitivity; for example, Hinnebusch and Schwan (7) could detect 10 cells in a spiked flea homogenate by using *pla* gene primers. We are continuing efforts to improve the sensitivity of our assay. One strategy would be to utilize probes directed against multiple-copy targets, such as 16S rRNA genes. Alternatively, it may be possible to integrate the 5′ nuclease assay into a heminested PCR protocol.

When the assay was tested with 43 different bacterial isolates, only *Y. pestis* isolates were positive. Strains pestoides Ba, C, and E gave negative results. The pestoides E results were expected because this strain lacks the *pla* gene (21). However,

the pestoides Ba and C results were not expected because these strains contain the *pla* gene. When the *pla* fragments from these strains were sequenced, they showed perfect homology with the primers and the probe. Therefore, they were retested three additional times with the TaqMan assay and gave positive signals with Ct values of <25.

We found that PCI-extracted DNA from the monkey tissues gave the most accurate results for the TaqMan 5′ nuclease assay. Two rounds of PCI extraction were necessary for detection of *Y. pestis* in the swab samples. As with the flea specimens (see below), substances present in the blood or tissues may have interfered with our PCR reagents, which would have required more laborious purification procedures. These extended procedures are a cause of some concern to us, as the extra time involved may negate the advantages offered by real-time detection systems, as well as the difficulties inherent in requiring facilities to handle and dispose of toxic solvents in the field. We are actively investigating new sample preparation methods in an attempt to address this issue.

When blood, serum, and oropharyngeal swabs from three infected monkeys were assayed, six (66%) of nine specimens were positive. In the original study, cardiac blood from all monkeys was culture positive for *Y. pestis* (5). We chose not to apply these results to our assay to calculate the epidemiologic sensitivity and specificity of the assay, as we cannot be sure that the irradiation and multiple freeze-thawing of the samples compromised their ability to serve as templates for PCR.

All monkeys had swab samples positive for *Y. pestis* (Fig. 2), indicating that the TaqMan 5′ nuclease assay was able to detect *Y. pestis* in these easily obtainable samples. Presumably, in a situation with personnel exposed to aerosolized formulations of plague bacilli, oral or nasal swabs would be among the specimens submitted for testing. The specimens we examined were necessarily obtained from experimental infections in which monkeys were exposed to aerosolized preparations in a controlled laboratory setting. Consequently, the bacterial concentration in these animals (which all succumbed to infection) was high and increased the likelihood of detecting the bacterial template in specimens.

We assayed experimentally infected fleas for *Y. pestis* by using the 5′ nuclease PCR assay. The best results (62%) were obtained when flea homogenates were subjected to two rounds of PCI extraction. In the conventional PCR assays reported by Hinnebusch and Schwan (7, 8), 90% of engorged fleas were positive; preparation of the fleas was simple, involving homogenization in 50 μl of brain heart infusion broth, heating at 95°C for 10 min, and then using 2 μl of supernatant as the template. This technique was more sensitive than more involved sample preparation methods (7). We attempted to use this protocol, substituting 25 μl of phosphate-buffered saline for brain heart infusion broth, but none of the samples were positive. It was necessary to perform at least one round (preferably two rounds) of PCI-mediated DNA extraction on the fleas to detect any bacteria. It may be that substances present in the flea, or in the ingested blood, inhibited the performance of our primers, probe, and/or reagents. The use of at least one round of PCI extraction to obtain an amplifiable template was reported for other arthropod-borne bacterial and viral pathogens (11, 17).

Our intent was not so much to improve upon existing PCR assays for *Y. pestis* detection in fleas and clinical specimens as to develop a method that provides high throughput and rapid turnaround and can be adaptable to use in the field. Non-nucleic-acid-based techniques are being developed for field use; for example, a fiber optic biosensor-based assay was tested for *Yersinia* detection (3) and a matrix-assisted laser desorption ionization time-of-flight mass spectrometry assay was developed (12). Our 5′ nuclease PCR assay is sensitive (picogram level of detection) and specific (only *Y. pestis* will react with the probe), is equivalent to existing PCR assays, and permits investigators to obtain results more quickly than with traditional techniques. We have preliminary results indicating that the *Y. pestis* TaqMan probe performs well on clinical samples when used with a portable analytical thermal cycling device (16). Therefore, our next goal is to adapt the 5′ nuclease PCR assay to these field-oriented instruments, providing medical personnel with real-time nucleic acid detection capability in environments where such capacities would be advantageous.

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