Yersinia pestis is a tier 1 agent due to its contagious pneumopathogenicity, extremely rapid progression, and high mortality rate. As the disease is usually fatal without appropriate therapy, rapid detection from clinical matrices is critical to patient outcomes. We previously engineered the diagnostic phage ΦA1122 with luxAB to create a “light-tagged” reporter phage. ΦA1122::luxAB rapidly detects Y. pestis in pure culture and human serum by transducing a bioluminescent signal response. In this report, we assessed the analytical specificity of the reporter phage and investigated diagnostic utility (detection and antibiotic susceptibility analysis) directly from spiked whole blood. The bioreporter displayed 100% (n = 59) inclusivity for Y. pestis and consistent intraspecific signal transduction levels. False positives were not obtained from species typically associated with bacteremia or those relevant to plague diagnosis. However, some non-pestis Yersinia strains and Enterobacteriaceae did elicit signals, albeit at highly attenuated transduction levels. Diagnostic performance was assayed in simple broth-enriched blood samples and standard aerobic culture bottles. In blood, <10^5 CFU/ml was detected within 5 h. In addition, Y. pestis was identified directly from positive blood cultures within 20 to 45 min without further processing. Importantly, coincubation of blood samples with antibiotics facilitated simultaneous antimicrobial susceptibility profiling. Consequently, the reporter phage demonstrated rapid detection and antibiotic susceptibility profiling directly from clinical samples, features that may improve patient prognosis during plague outbreaks.
rapidly transduce a bioluminescent phenotype (≤20 min) and sensitively detect *Y. pestis* in pure culture and in spiked human serum, thereby displaying promise as a diagnostic tool. In addition, as phage infection and gene expression are proportional to cell fitness, the reporter phage can rapidly generate antimicrobial susceptibility data from pure cultures, which correlate closely to results obtained using standard methods (14). In this report, we examine the diagnostic performance of the *Y. pestis* reporter phage directly from blood and from commercial blood culture bottles and evaluate critical diagnostic parameters.

**MATERIALS AND METHODS**

**Bacterial strains.** *Bacillus anthracis* (attenuated strain) was kindly provided by the CDC, Atlanta, GA (Elke Saile), *Escherichia coli* (5 various O-antigenic and uropathogenic strains), *Enterococcus faecalis* (5 strains), *Enterococcus faecium* (2 strains), *Klebsiella pneumoniae* (2 strains), *Francisella tularensis* (2 attenuated strains), *Klebsiella oxytoca* (1 strain), *Klebsiella* spp. (2 strains not identified to species level), *Listeria monocytogenes* (5 strains), *Salmonella enterica* (10 strains), *Shigella flexneri* (3 strains), *Shigella boydii* (1 strain), *Shigella sonnei* (3 strains), *Staphylococcus aureus* (3 strains), *Staphylococcus epidermidis* (2 strains), *Streptococcus pneumoniae* (3 strains), *Yersinia enterocolitica* (10 strains), *Yersinia pseudotuberculosis* (10 strains), and the attenuated *Y. pestis* strain A1122 were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) or the American Type Culture Collection (ATCC). Experiments involving virulent *Y. pestis* strains were performed at the University of Florida under biosafety level 3 (BSL3) conditions. A collection of 58 strains, including the classical strains KIM and CO92 as well as strains isolated from diverse sources (human, rodents, and fleas) and different geographical locations (e.g., United States, former Soviet Union, Brazil, Zimbabwe, India, and Nepal), were analyzed. A list of strains is provided in Tables S1 to S4 in the supplemental material.

**Phage.** The *Y. pestis* diagnostic phage ΦA1122::luxAB was described previously (14). Reporter phage stocks were prepared from *Y. pestis* A1122 using agar overlays. Phages were eluted from the top agar in SM buffer, clarified by centrifugation (4,000 × g for 10 min), and vacuum-filter sterilized (0.22 μM twice before treatment with 1 M NaCl and precipitation with 8% polyethylene glycol (BioUltra 8000; Sigma-Aldrich, St. Louis, MO). The phage precipitate was resuspended in ~1/10 original volume of SM buffer and adjusted to a concentration of 1 × 10^10 to 1 × 10^11 PFU/ml. Filter sterilization was repeated for concentrated preparations and confirmed by negative culture on Luria-Bertani (LB) agar. Preparations were stored at 4°C.

**Bacterial growth and manipulation.** Frozen stocks (in 25% glycerol at ~70°C) of *Y. pestis* A1122 and the 58 virulent strains were individually streaked onto brain heart infusion (BHI) agar with 6% sheep blood agar (SBA) and LB agar, respectively, and incubated for 48 h at 28°C. Isolates were subcultured in LB broth at 28°C with shaking (250 rpm). Assays were performed using early-exponential-phase cells (A_600 of ~0.2). *B. anthracis*, *E. coli*, *L. monocytogenes*, *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Enterococcus* and *Staphylococcus* spp. were cultured using BHI medium at 37°C. *Klebsiella*, *Salmonella*, and *Shigella* spp. were cultured in tryptic soy media, and *F. tularensis* strains were cultured on BHI or cystine heart agar (CHA) supplemented with 2% hemoglobin. Cultures at an A_600 of 0.2 ± 0.03 were used for experiments. All manipulations were performed within a class II biosafety cabinet.

**Bioluminescence assays directly from blood and blood culture bottles.** Fresh (≤1-week-old) whole human blood collected with sodium citrate (Research Blood Components, LLC, Boston, MA) was spiked with *Y. pestis* A1122 at the indicated concentrations (see Fig. 2 legend). Spiked blood was diluted 1:20 with LB (50 μl into 950 μl) in 14-ml culture tubes and incubated at 28°C with shaking (250 rpm). After 4 h of incubation, blood cells were collected by centrifugation (250 × g for 1 to 2 min), and the resulting supernatants were extracted and recentrifuged at 10,000 × g for 4 min. Bacterial cell pellets were resuspended in 225 μl of remaining supernatant and mixed with the ΦA1122::luxAB reporter phage (1.1 × 10^9 PFU/assay).

Spiked blood samples were also syringe transferred (5 ml) into blood culture bottles (BacT/Alert Standard Aerobic [bioMérieux, Inc., Durham, NC] or Bactec Plus Aerobic/F culture vials [BD and Co., Sparks, MD]) (final blood dilution factor, 1/9 in BacT/Alert and 1/7 in Bactec bottles). Spiked samples were incubated at 35 to 37°C within BacT/Alert and Bac- tec blood culture systems. Aliquots from seeded (~10^3-CFU/ml) culture system-positive Bactec bottles were also assayed without the need for the aforementioned processing and concentration steps. Phage-infected samples were incubated with shaking (250 rpm) for 20 to 45 min. Aliquots (195 μl) were added to 96-well microtiter plates and measured for bioluminescence (GloMax 96 Microplate Lumimeter 9101-001; Promega Corp., Madison, WI) for 10 s following injection of 2% β-naphthaldehyde.

**Antibiotic resistance/susceptibility profiling from blood.** To assess the ability of the reporter phage to detect and simultaneously determine antibiotic susceptibility of *Y. pestis* in blood, spiked blood samples were mixed with LB containing chloramphenicol, streptomycin, or tetracycline at increasing concentrations throughout known activity ranges and MICs. Samples were treated as described above for bioluminescence assays directly from blood. Antibiotic efficacy was confirmed against the quality control strain *S. aureus* strain ATCC 29213 according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution methods (7, 8).

**Specificity and selectivity assays.** Selectivity of the recombinant ΦA1122::luxAB reporter was assayed for bioluminescence against 59 strains of *Y. pestis*. Recombinant phage specificity was also determined against 47 strains of closely related genera (i.e., *Enterobacteriaceae*) and 26 strains of nonrelated but clinically relevant bacterial pathogens through bioluminescence assays. Briefly, log-phase cultures (1 ml) were plated into 14-ml snap-cap tubes (n = 3), infected with ΦA1122::luxAB, and assayed for bioluminescence over time. Relative bioluminescence (RB) was calculated by dividing the average relative light units (RLU) of *Y. pestis* A1122 by the average RLU of the test strain. Efficiency of plating (EOP), as defined by the phage titer on a test strain/species compared to the maximum titer, was also analyzed where appropriate using *Y. pestis* A1122 as the standard (12). Specific strain data for EOP and relative bioluminescence are provided in Tables S2 and S3 in the supplemental material.

**Statistics.** Reported as CFU/ml or PFU/ml, all bacterial cell or phage titer concentrations were quantified as the averages of duplicate colony or plate count plates, respectively. Bioluminescence values, presented as relative light units (RLU), are the averages of three infections (n = 3) ± standard deviations (SD). All experiments were performed in duplicate. Statistical significance was determined using unpaired Student’s t tests. Tukey’s adjusted analyses of variance (ANOVA) were performed for comparisons between multiple data sets, and Pearson’s correlation coefficient (r) was used to evaluate dependencies between data sets. Significance was assigned at P ≤ 0.05. Statistical analyses were performed using Microsoft Excel 2010 and GraphPad Prism 5.0 on Windows 7.

**RESULTS**

**Selectivity and specificity.** We previously analyzed the ability of ΦA1122::luxAB to detect *Y. pestis* A1122, an attenuated exempt select agent strain. However, the functionality of the reporter phage against a range of virulent strains had not been assessed. Therefore, performance was tested against a diverse library of *Y. pestis* strains (n = 59). In addition, non-*Y. pestis* Yersinia spp., other *Enterobacteriaceae*, and distantly related but clinically relevant bacterial pathogens were also analyzed. Of the 59 *Y. pestis* strains, all (100%) elicited rapid bioluminescence of similar signal intensities and kinetics (Fig. 1 and data not shown). For example, after 20 min of ΦA1122::luxAB infection, *Y. pestis* CO92 elicited a 100-fold increase in signal compared to controls, with peak light signal manifesting at 40 to 50 min postinfection (Fig. 1). Dose-response analysis of 10-fold serially diluted cells indicated that a
bacteremia. Therefore, the efficacy of reporter phage-mediated detection of Y. pestis A1122 was assessed directly from blood and blood culture bottles. Y. pestis was detected in diluted blood within 5 h, without the need for culture isolation (Fig. 2A, P < 0.05). The signal response exhibited dose-dependent characteristics with a sensitivity limit of detection (LoD) in the 10^2 CFU/ml range, peaking 45 min after phage infection. Similar results were obtained from BacT/Alert and Bectec culture bottles, indicating a functional compatibility of this detection platform with established clinical procedures for processing blood samples. Although “light” signal measurements were somewhat attenuated in blood, signal production was extremely rapid. Seeded Bectec bottles (n = 6) registered positive for bacterial growth after 24 to 30 h (blood-alone controls did not register positive as expected), and aliquots from these bottles were diagnosed by the reporter phage 20 to 45 min after infection without sample processing or concentration (Fig. 2B and C, P < 0.05).

**Rapid antibiotic susceptibility profiling from blood.** Antimicrobial susceptibility profiling using the reporter phage has been previously demonstrated using pure cultures (14). To assess the ability of ΦA1122::luxAB to profile antibiotic susceptibility of Y. pestis without the need for culture isolation, blood samples were spiked with ca. 10^2 CFU/ml Y. pestis and diluted in LB medium containing increasing concentrations of chloramphenicol, streptomycin, or tetracycline along the known MIC ranges (Fig. 3A to C). These antibiotics were selected because they are the standard therapeutics and prophylactics recommended for plague in hu-

### TABLE 1 Specificity of ΦA1122::luxAB among non-pestis Yersinia species, closely related species, and nonrelated but clinically relevant bacterial pathogens

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>No. of positive strains/total no. tested</th>
<th>Relative bioluminescence (range)</th>
<th>Efficiency of plating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yersinia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>3/10</td>
<td>10^6–10^4</td>
<td>≤3.6 × 10^-6</td>
</tr>
<tr>
<td>Y. pseudotuberculosis</td>
<td>7/10</td>
<td>10^6–10^1</td>
<td>&lt;1 × 10^-6</td>
</tr>
<tr>
<td>Entero bacteriae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>0/5</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>0/5</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
<tr>
<td>S. enterica</td>
<td>4/10</td>
<td>10^4–10^5</td>
<td>&lt;1 × 10^-6</td>
</tr>
<tr>
<td>S. boydii</td>
<td>0/1</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
<tr>
<td>S. flexneri</td>
<td>0/3</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>2/3</td>
<td>10^6–10^5</td>
<td>≤1.2 × 10^-5</td>
</tr>
<tr>
<td>Non-Enterobacteriae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. anthracis</td>
<td>0/1</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>0/5</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
<tr>
<td>E. faecium</td>
<td>0/2</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
<tr>
<td>F. tularensis</td>
<td>0/2</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>0/5</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
<tr>
<td>Staphylococcus spp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>0/3</td>
<td>Background (ND)</td>
<td>5</td>
</tr>
</tbody>
</table>

**a** Relative to Y. pestis A1122; relative bioluminescence = RLU of non-Y. pestis strain/RLU of Y. pestis A1122 at 28°C.

**b** Efficiency of plating relative to Y. pestis A1122 at 28°C. ND, not determined.

**c** Includes O91:H21, O157:H7, O145:NM, and a uropathogenic strain (serotype unknown).

**d** Includes K. oxytoca and K. pneumoniae.

**e** Includes both Typhi and non-Typhi Salmonella enterica subsp. enterica strains.

**f** Includes S. aureus and S. epidermidis.
Samples were incubated for 4 h, infected with $Y.\ pestis$ A1122::luxAB, and then assayed for bioluminescence after 45 min. For each antibiotic, bioluminescence was inversely proportional to antibiotic concentration. High antibiotic concentrations completely nullified signal production, whereas low antibiotic concentrations resulted in strong signals. Thus, rapid detection and simultaneous antibiotic susceptibility profiling of low levels of $Y.\ pestis$.

**FIG 2** Bioluminescent detection of $Y.\ pestis$ from whole human blood. (A) One-milliliter blood aliquots harboring $Y.\ pestis$ A1122 ($5.5 \times 10^4$ to $5.5 \times 10^5$ CFU/ml) were diluted 1:20 in LB, enriched for 4 h, and analyzed 45 min after phage infection for a bioluminescent signal response. (B) Blood (5 ml) was spiked with 12 CFU/ml $Y.\ pestis$ A1122 and transferred to standard aerobic BacT/Alert culture bottles. Seeded bottles were machine (BacT/Alert 3D 60) incubated until a colorimetric positive reading was obtained (24 to 27 h). Neat or diluted aliquots were analyzed 20 and 45 min after phage infection for a bioluminescent signal response. (C) Blood (5 ml) was spiked with 4.1 CFU/ml $Y.\ pestis$ A1122 and transferred to Plus Aerobic/F Bactec culture bottles. Bottles were machine incubated until a colorimetric positive reading was obtained (24 to 30 h). Neat or diluted aliquots were analyzed 20 and 45 min after phage infection for a bioluminescent signal response. Values are means ± SD from 3 independent spiked blood samples. Graphs presented are representative of 2 independent experiments. Asterisks denote a significant difference increase ($P < 0.05$) between the assigned value and the next highest antibiotic concentration.

**FIG 3** Phage-mediated antibiotic susceptibility profiles of $Y.\ pestis$ A1122 directly from blood incubated in the presence of chloramphenicol (A), streptomycin (B), or tetracycline (C). Antibiotics were prepared using the CLSI microdilution method. Whole blood was spiked with 75, 44, and 105 CFU/ml for panels A, B, and C, respectively; dilute 1:20; and incubated at $35^\circ C$ for 4 h before being infected with the reporter phage. Values are means ± SD from 3 independent infections of spiked blood. Graphs presented are representative of 2 independent experiments. Asterisks denote a significant increase ($P < 0.05$) compared to phage-negative controls (cells alone in blood).
**DISCUSSION**

Plague is definitively diagnosed by the isolation and identification of the organism from clinical specimens or by demonstrating a 4-fold or greater change in antibody titer against the F1 antigen in paired serum specimens. As phage A1122 can be used to identify *Y. pestis* using classical lysis assays, A1122::luxAB has the potential to expedite this process. Data obtained using strain A1122 indicated that detection was achieved with blood spiked with 100 CFU/ml within 5 h, with similar results from blood culture. Of note, samples from culture-positive blood bottles were “diagnosed” by the reporter with no preparation, processing, or concentration steps, within 20 to 45 min. Because massive plague bacteremia generally foreshadows a fatal prognosis, and bacteremic concentrations of ≥100 CFU/ml are significantly correlated with mortality, detection of plague bacilli below this level is crucial for a clinically actionable plague diagnostic (6, 15, 16).

A1122::luxAB efficiently detected all *Y. pestis* strains tested and likely possesses the same specificity as its parent phage, which infects all but two of thousands of isolates. However, the broad intra-specific host range is counterbalanced by suboptimal cross-reactivity, as the reporter transduced bioluminescence to 16 of 47 strains of phylogenetically related species (i.e., *Enterobacteriaceae*). In cross-reactive strains, amplitude of transduced light was 10^{-1} to 10^{6}-fold lower (overall median RB = 1.13 × 10^{-3} RLU/assay) and time to peak signal was generally longer than those for *Y. pestis*. In receptive *Y. pseudotuberculosis* strains, assays at subdiagnostic temperature (20°C) revealed signal attenuation by up to 10^{4}-fold in most, but not all, strains evaluated. Therefore, the reporter phage may require complementary diagnostic testing to reduce the likelihood of false positives, and particularly to discriminate between *Y. pestis* and *Y. pseudotuberculosis*. Importantly, none of the assayed pathogens integral in the differential diagnosis for plague (i.e., *B. anthracis*, *F. tularensis*, and *Klebsiella pneumoniae*) or those most frequently associated with bacteremia (i.e., *Enterococcus spp.*, *E. coli*, coagulase-negative *Staphylococcus spp.*, and *Streptococcus spp.*) (17–19) were positive for bioluminescence. However, as the reporter phage diagnostic requires laboratory equipment and up to 5 h to elicit a response from clinical specimens, it is not usable in its current form as a point-of-care assay. In contrast, the plaque dipstick tests which target the F1 antigen are specific (no other yersinia are detected) and sensitive and are particularly useful for field applications as they are rapid (less than 15 min) and do not require equipment (20, 21). Therefore, dipstick tests have many advantages over the reporter phage technology. However, as F1-negative isolates can occur, albeit rarely (22), the reporter phage may be a useful alternative to tests that rely on the F1 antigen.

Appropriate antibiotic therapy within 18 to 24 h of symptom onset is essential for a positive prognosis in plague patients (23, 24). Chloramphenicol, streptomycin, and tetracycline are the recommended primary-level treatments (1, 6). In addition to the potential for engineered resistance in bioweapons, naturally occurring antibiotic-resistant strains of *Y. pestis*, although extremely rare, have been isolated (2, 3, 25). Thus, the ability to rapidly generate an antibiotic susceptibility profile is of value. Immunoassays and most PCR-based methods are unable to distinguish viable from dead cells and are therefore of limited use in determining antibiotic susceptibility. A method employing flow cytometry to determine antibiotic susceptibility following the separation of plague bacilli from spiked blood cultures has been described, although the need for preenrichment extends the assay time to ca. 39 h. The reporter phage diagnostic described here has the ability to detect *Y. pestis* and to simultaneously determine its susceptibility to antibiotics directly from blood within 5 h. This diagnostic therefore has the potential to provide critical information, timely enough to augment treatment and improve patient outcomes in both bioterrorism and naturally acquired cases of plague.

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**REFERENCES**


23. Woods JB. 2005. USAMRIID’s management of biological casualties handbook. USAMRIID, Frederick, MD.
