Rapid detection and simultaneous antibiotic susceptibility analysis of *Yersinia pestis* directly from clinical specimens using reporter phage

Vandamm, J. P\(^1\), Rajanna, C\(^2\), Sharp, N. J\(^1\), Molineux, I. J\(^3\), and Schofield, D. A\(^1\#\)

\(^1\)Guild BioSciences, Charleston, South Carolina, 29407

\(^2\)Department of Molecular Genetics and Microbiology, University of Florida, Gainesville 32610

\(^3\)Molecular Biosciences, Institute for Cellular and Molecular Biology, University of Texas at Austin, Texas, 78712

\(^\#\)Corresponding author. Mailing address: Guild BioSciences, 1313B Ashley River Road, Charleston, SC 29407. Phone: (843) 573 0095. Fax: (843) 573 0707.

E-mail: dschofield@guildbiosciences.com

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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 investigate diagnostic utility (detection and antibiotic susceptibility analysis) directly from spiked whole blood. The bioreporter displayed 100% (n=59) inclusivity for Y. pestis and consistent intra-specific 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, <10^2 CFU/mL was detected within 5 h. In addition, Y. pestis was identified directly from positive blood cultures within 20-45 min without further processing. Importantly, co-incubation 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.
INTRODUCTION

*Yersinia pestis*, the causative agent of the plague, is a Tier 1 agent carrying great risk for deliberate misuse and a significant socio-economic, public health threat. Plague has the ability to spread person-to-person and is typically fatal in the absence of expedient antibiotic therapy. Over the last 20 years, there has been a global rise in plague incidence and strains have been isolated from patients with bubonic plague that are streptomycin- and multidrug-resistant (MDR) (1-3). However, *Y. pestis* is typically not drug resistant, and recent data indicates pneumonic plague may not be as transmissible as previously believed (4). A zoonotic pathogen, *Y. pestis* circulates among rodents and lagomorph populations, with endemic foci found in Asia, Africa, the Americas, and the former Soviet Union. Most commonly spread to humans by fleas, plague typically begins by bacterial multiplication at the site of dermal inoculation, followed by spread to the draining lymph node via lymphatic vessels, and the formation of characteristic buboes. Bubonic plague can rapidly progress to secondary septicemia and in some cases, the bacteria can spread systemically and infiltrate alveoli, resulting in the highly lethal pneumonic form. Because of the potential for pneumonia ensuing from bubonic or septicemic plague, the CDC recommends quarantine of all plague patients (5). Of greatest concern in bioterrorism is primary pneumonic plague contracted via inhalation of aerosolized bacilli, which frequently leads to septicemia. Pneumonic plague can be spread person-to-person and, like septicemic plague, is nearly always fatal without appropriate antibiotic therapy and aggressive supportive care within 18 to 24 h of symptom onset (6).
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 titre against the F1 antigen in paired serum specimens. Because *Y. pestis* grows slowly (doubling time of 1.25 h at 28°C), culturing from clinical specimens can involve incubation for 24 to 72 h. Antibiotic susceptibility analysis from colony isolates requires an additional 24 to 48 h (7, 8) and thus can extend the total diagnostic timeline to between 2 and 5 days. However, because of the fulminant nature of plague infection, rapid appropriate antibiotic therapy is paramount to patient prognosis. Therefore, faster methods of detection and antimicrobial susceptibility profiling of *Y. pestis* directly from clinical matrices may be of valuable.

Classical phage lysis assays using the plague diagnostic phage ΦA1122 are utilized by the WHO, CDC and public health laboratories for *Y. pestis* identification. Thought to target receptors likely essential for growth and virulence (9-11), ΦA1122 infects and lyses all but two out of thousands of *Y. pestis* strains (12). In order to increase the time to detection and bypass the necessity for pure cultures, ΦA1122 was previously genetically engineered with the genes encoding bacterial luciferase (*luxA* and *luxB*) to create a reporter phage capable of transducing bioluminescence to infected cells (13). The reporter phage ΦA1122::*luxAB* was able to 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 is 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
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 (Dr. Eilke 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 unspeciated strains), *Listeria monocytogenes* (5 strains), *Salmonella enterica* (10 strains), *Shigella flexneri* (3 strains), *Shigella boydii* (1 strain), *Shigella sonnei* (3 strains), *Staphylococcus aureus* (4 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 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. U.S., former Soviet Union, Brazil, Zimbabwe, India and Nepal) were analyzed. A list of strains is provided in Supplementary Tables S1-S4.

**Phage.** The *Y. pestis* diagnostic phage ΦA1122::luxAB was described previously (14). Reporter phage stocks were prepared from *Y. pestis* A1122 using agar overlays.
Phage were eluted from the top agar in SM buffer, clarified by centrifugation (4,000 x g for 10 min) and vacuum-filter sterilized (0.22 μM) twice before treating with 1M NaCl and precipitating with 8% polyethylene glycol (BioUltra 8000, Sigma-Aldrich, St. Louis, MO). The phage precipitate was resuspended in ~1/10th original volume SM buffer and adjusted to a concentration of 1 x 10^10 to 10^11 plaque forming units (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 media at 37°C. *Klebsiella*, *Salmonella* and *Shigella* spp. were cultured in Tryptic Soy media, and *F. tularensis* strains 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 a 4
h incubation, blood cells were collected by centrifugation (250 x g for 1 to 2 min) and the resulting supernatants extracted and re-centrifuged at 10,000 x 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 x 10⁹ PFU/assay).

Spike 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®, 1:7 in Bactec™ bottles). Spiked samples were incubated at 35 to 37°C within BacT/Alert® and Bactec™ Blood Culture Systems. Aliquots from seeded (~10¹ 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 Luminometer 9101-001, Promega Corp., Madison, WI) for 10 s following injection of 2% n-decanal.

**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 minimum inhibitory concentrations (MIC). Samples were treated as described above for bioluminescence assays directly from blood. Antibiotic efficacy was confirmed against the quality control *S. aureus* strain ATCC29213 according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (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 non-related but clinically relevant bacterial pathogens through bioluminescence assays. Briefly, log-phase cultures (1 mL) were placed 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 is provided in Supplementary Tables S2 and S3.

Statistics. Reported as CFU/mL or PFU/mL, all bacterial cell or phage titer concentrations were quantified as the average of duplicate colony or plaque plate counts, respectively. Bioluminescence, 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 ANOVAs 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, 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 60 min post infection (Fig. 1). Dose response analysis of 10-fold serially diluted cells indicated that a bioluminescent signal (RLU) was detectable from 2400, 240, and 24 CFU/mL after 20, 40, and 60 min of infection, respectively (Fig. 1, p<0.05). Similar data was obtained with 4 other *Y. pestis* strains (A1122, 2095G, NR-20, and NR-641). The data also indicates that: (i) ΦA1122::luxAB exhibits the same broad intra-species as its parent ΦA1122, and (ii) results obtained using the attenuated *Y. pestis* A1122 strain are translatable to virulent strains.

The reporter phage was challenged against 73 non-*Y. pestis* strains for phage-mediated bioluminescence, and in some cases, for phage amplification (EOP assays). These included closely related Yersiniae, members of the Enterobacteriaceae, *enterococci* and *staphylococci* and *B. anthracis* and *F. tularensis*. Three of 10 *Y. enterocolitica* strains and 7 of 10 *Y. pseudotuberculosis* strains elicited phage-mediated bioluminescence (Table 1). Of these, only one *Y. pseudotuberculosis* strain expressed a signal as high as one log below that of *Y. pestis*, while the remainder were generally 10^4 lower.
to 10^5-fold lower. In general, there was a correlation between phage-mediated bioluminescence and EOP; strains that elicited low bioluminescence yielded low EOP.

Due to temperature-dependent differences in LPS structure, ΦA1122 does not form plaques on *Y. pseudotuberculosis* below 28°C (9, 12). As expected, phage-mediated bioluminescence assays at 20°C revealed an attenuated (10^2 to 10^4-fold lower) signal in 5 *Y. pseudotuberculosis* strains compared to assays performed at 37°C. Unexpectedly, one strain elicited a 10^3-fold increase at the reduced temperature (data not shown).

Among other species, none of the *E. coli, Klebsiella, S. boydii* or *S. flexneri* strains elicited phage-mediated bioluminescence (Table 1). However, bioluminescence was detected from four *S. enterica* and two *S. sonnei* strains, although the signal response was extremely low. As may be expected, none of the phylogenetically distant but clinically relevant strains elicited reporter phage-mediated bioluminescence.

**Rapid detection of *Y. pestis* from blood and blood culture bottles.** Because bacteremia is common in bubonic, pneumonic and septicemic plague, blood is an important matrix for laboratory diagnosis. Blood culture systems can be used to screen for 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 Bactec™ 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 Bactec™ 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 & 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 containing increasing concentrations of chloramphenicol, streptomycin, or tetracycline along the known MIC ranges (Fig. 3A-C). These antibiotics were selected because they are the standard therapeutics and prophylactics recommended for plague in humans (6). Samples were incubated for 4 h, infected with ΦA1122::luxAB, and then assayed for bioluminescence after 45 min. For each antibiotic, bioluminescence was inversely proportional to antibiotic concentration. High antibiotic concentrations completely nulified signal production whereas low antibiotic concentrations resulted in strong signals. Thus, rapid detection and simultaneous antibiotic susceptibility profiling of low levels of Y. pestis is achieved through use of the reporter phage detection platform.

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 titre
against the F1 antigen in paired serum specimens. As phage A1122 can be used to identify *Y. pestis* using classical lysis assays, \( \Phi A1122:\::\luxAB \) has the potential to expedite this process. Data obtained using strain A1122 indicated 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 \( \geq 100 \) 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).

\( \Phi A1122:\::\luxAB \) efficiently detected all *Y. pestis* strains tested, and likely possesses the same specificity of its parent phage, which infects all but two of thousands of isolates. However, the broad intraspecific 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 to \( 10^8\)-fold lower (overall median \( RB = 1.13 \times 10^{-5} \)) RLU/assay and time to peak signal generally longer than for *Y. pestis*. In receptive *Y. pseudotuberculosis* strains, assays at sub-diagnostic 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 test. In contrast, the plague dipstick tests which targets the F1 antigen are specific (no other Yersiniae are detected), 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 pre-enrichment 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.

ACKNOWLEDGEMENTS

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Table 1: Specificity of ΦA1122::luxAB among non-pestis Yersiniae, closely-related species and non-related but clinically relevant bacterial pathogens.

<table>
<thead>
<tr>
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<th>Positive strains/total tested</th>
<th>Relative bioluminescence (range)$^a$</th>
<th>Efficiency of plating$^b$</th>
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<tr>
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<td>$10^{-6}$ to $10^{-4}$</td>
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<td>Y. pseudotuberculosis</td>
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<td>S. pneumoniae</td>
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$^a$ Relative bioluminescence expressed in arbitrary units (AU).

$^b$ Efficiency of plating expressed as the ratio of plating efficiency (PE) of the test strain to that of the control strain (P).
aRelative to *Y. pestis* A1122; relative bioluminescence (RB) = RLU of non-*Y. pestis* strain / RLU *Y. pestis* A1122 at 28°C.

bEfficiency of plating (EOP), relative to *Y. pestis* A1122 at 28°C.

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

dIncludes *K. oxytoca* and *K. pneumoniae*.

eIncludes both Typhi and non-Typhi *S. enterica* subspecies *enterica* strains.

f Includes *S. aureus* and *S. epidermidis*.

nd, not determined
FIG. 1. Bioluminescent response kinetics for the detection of *Y. pestis* CO92. Actively growing (*A*$_{600}$ of ~ 0.2) cells were serially diluted, infected with 1.4 x 10$^8$ PFU/mL ΦA1122::luxAB and assayed for bioluminescence (RLU) following the addition of *n*-decanal substrate. (*) denotes a significant increase (p < 0.05) compared to phage-negative (cells only) controls at the designated times. Values are means (n=3) ± SD. Similar results were obtained with 58 other *Y. pestis* strains.

FIG. 2. Bioluminescent detection of *Y. pestis* from whole human blood.

A. 1 mL blood aliquots harboring *Y. pestis* A1122 (5.5 x 10$^6$ to 5.5 x 10$^2$ CFU/mL), were diluted 1:20 in LB, enriched for 4 h, and analyzed 45 min post 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 (24 to 27 h). Neat or diluted aliquots were analyzed 20 and 45 min post 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 (24 to 30 h). Neat or diluted aliquots were analyzed 20 to 45 min post 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 increase ($p < 0.05$) compared to phage negative controls (cells alone in blood).

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 A, B, and C, respectively, diluted 1:20 and incubated at 35°C for 4 h before being infected with the reporter phage. Values are means ± SD from 3 independent infections of spiked blood. Graph 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.
REFERENCES


