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A non-invasive *in vivo* imaging system to study dissemination of bioluminescent *Yersinia pestis* CO92 in a mouse model of pneumonic plague

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ABSTRACT

The gold standard in microbiology for monitoring bacterial dissemination in infected animals has always been viable plate counts. This method, despite being quantitative, requires sacrificing the infected animals. Recently, however, an alternative method of *in vivo* imaging of bioluminescent bacteria (IVIBB) for monitoring microbial dissemination within the host has been employed. *Yersinia pestis* is a Gram-negative bacterium capable of causing bubonic, septicemic, and pneumonic plague. In this study, we compared the conventional counting of bacterial colony forming units (cfu) in the various infected tissues to IVIBB in monitoring *Y. pestis* dissemination in a mouse model of pneumonic plague. By using a transposon mutagenesis system harboring the luciferase (*luc*) gene, we screened approximately 4000 clones and obtained a fully virulent, *luc*-positive *Y. pestis* CO92 (*Y. pestis-luc2*) reporter strain in which transposition occurred within the largest pMT1 plasmid which possesses murine toxin and capsular antigen encoding genes. The aforementioned reporter strain and the wild-type CO92 exhibited similar growth curves, formed capsule based on immunofluorescence microscopy and flow cytometry, and had a similar LD_{50}. Intranasal infection of mice with 15 LD_{50} of CO92-*luc2* resulted in animal mortality by 72 h, and an increasing number of bioluminescent bacteria were observed in various mouse organs over a 24–72 h period when whole animals were imaged. However, following levofloxacin treatment (10 mg/kg/day) for 6 days 24 h post infection, no luminescence was observed after 72 h of infection, indicating that the tested antimicrobial killed bacteria preventing their detection in host peripheral tissues. Overall, we demonstrated that IVIBB is an effective and non-invasive way of monitoring bacterial dissemination in animals following pneumonic plague having strong correlation with cfu, and our reporter CO92-*luc2* strain can be employed as a useful tool to monitor the efficacy of antimicrobial countermeasures in real time.

1. Introduction

Yersinia pestis, one of three human pathogenic *yersiniae* species, has a complicated life cycle occurring in both arthropod vectors and mammalian hosts (e.g. rats, squirrels, and prairie dogs). Unfortunately, the arthropod vector can transmit the pathogen to humans in close proximity to animal reservoirs [1]. The notorious pathogen is responsible for over 200 million deaths stemming from the three major human plague pandemics that it has caused [2,3]. In sharp contrast, the closely related pathogenic yersiniae, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* cause a relatively benign self-limiting gastro-intestinal disease in humans [4]. Today, plague continues to be a problem in endemic regions of India and China where rodent populations tend to be high. In fact, there are approximately 1000–2000 human infection cases globally with some instances of infection occurring in the four corners region of the United States (where Arizona, Colorado, New Mexico, and Utah meet) as well as in parts of California [5,6].

Presently, the World Health Organization has designated plague caused by *Y. pestis* as a re-emerging infectious disease [7,8]. More importantly, the weaponization of a multiple-drug resistant

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Y. pestis strain poses a significant health threat to society [9,10]. Since several clinical isolates of *Y. pestis* have naturally acquired resistance to chloramphenicol, streptomycin, and penicillin derivatives [11,12], recent studies have evaluated the efficacy of new classes of antimicrobials including the fluoroquinolone levofloxacin and a ketolide cethromycin in animal models of plague [13,14]. Even though both of the aforementioned drugs cleared the plague bacilli when treatment was initiated within 48 h of infection in a rat model of pneumonic plague [14], additional novel anti-plague drugs need to be developed, evaluated, and made available to the public. Only recently, levofloxacin was approved by the Food and Drug Administration (FDA) for plague, and this approval was based solely on animal efficacy data (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm302220.htm). Furthermore, many plague researchers are also alternatively working to develop an efficacious plague vaccine since currently no vaccine is available for human use [15].

Taking into consideration the threat of multiple-drug resistant *Y. pestis* in a biological warfare setting, against which we have no effective prophylactic vaccine or antimicrobials, a reporter strain that can provide real time dissemination information within a host could be a useful tool when testing the efficacy of novel chemotherapeutics. Similarly, such a tool would prove useful when holistically measuring the virulence potential of a live-attenuated vaccine candidate strain or to demonstrate protective efficacy of a subunit vaccine against plague. In fact, bioluminescent imaging (BLI) has been used for monitoring the dissemination of *Burkholderia* following a respiratory infection in mice [16]. While our studies were in progress, Nham et al. reported monitoring dissemination of *Y. pestis* in BALB/c mice in a bubonic plague model of infection [17]. In both instances, BLI allowed for real time, semi-quantitative visualization of bacterial loads in various tissues following bacterial dissemination from the sites of infection at multiple time points. BLI takes advantage of either ectopic, plasmid-driven or transposon-mediated, chromosomally-integrated expression of a luciferase (*luc*) gene [16].

In this study, we employed the transposon-encoded luminescence operon (pUTmini-Tn*5*::*lux*Km2) to generate our *Y. pestis* reporter strain, *Y. pestis-luc2*. The reporter strain did not suffer any attenuation in its virulence potential, and the *luc* gene was stably expressed following its insertion into the pMT1 plasmid. We then characterized bacterial dissemination of *Y. pestis-luc2* in Swiss Webster mice at various time points and determined the impact of levofloxacin treatment (24-h post infection) on bacterial dissemination using IVIBB.

2. Results

2.1. Generation and characterization of the Y. pestis-luciferase (luc) reporter strains

Since IVIBB has not been previously employed to monitor dissemination of *Y. pestis* CO92 following an intranasal challenge of mice, we sought to characterize the utility of such an approach in evaluating the efficacy of an antimicrobial treatment in preventing bacterial dissemination. Therefore, *Y. pestis* CO92 cells were transformed with pUTmini-Tn*5*::*lux*Km2 plasmid. We screened approximately 4,000 colonies, indicating either insertion of the transposon into bacterial chromosome or plasmids or the possibility of transposon harboring free plasmid in *Y. pestis*. Upon, *in vivo* image analysis of these clones, 5 colonies were initially bioluminescent, and 2 of them (*luc1* and *luc2*) exhibited strong bioluminescence with radiance above 5×10^6 ρ/s (i.e., total flux). These 2 clones were then used to intranasally challenge mice with a 15 LD_{50}. One clone (*luc2*) consistently exhibited better bioluminescence in animals compared to the other clone (*luc1*; data not shown).

We then examined these two clones to determine the site of transposon insertion. Within *luc2*, the transposon insertion was at 1020 base pairs (bp) downstream of the murine toxin gene (*ymt*) start codon (located within the pMT1 plasmid). Within *luc1,* the transposon insertion was 603 bp downstream of the long-chain fatty acid outer membrane transporter gene (*fadL*) start codon. The encoded FadL is essential for the uptake of long-chain fatty acids (C12 to C18) in *Escherichia coli* [18].

To ensure that the insertion of transposon harboring the *luc* gene did not disrupt the *caf1* (encoding capsular antigen F1, a virulence associated factor) open reading frame, we used anti-F1 primary antibodies followed by secondary antibodies conjugated to Alexa Fluor 488 or 594 prior to performing flow cytomteric/immunofluorescence analyses. Doing so enabled us to evaluate the presence of F1 antigen in both the *Y. pesitis-luc2* reporter and its parental CO92 strain. Relative to the isogenic parental strain, the reporter *Y. pestis-luc2* exhibited a very similar emission spectrum by fluorescent activated cell sorting (FACS) analysis, demonstrating that the *caf1* open reading frame was not disrupted by the integration of the *luc* gene into the pMT1 plasmid (Fig. 1A). The one advantage to employing FACS is that it allows for the analysis of individual cells; as a result, we have used FACS previously for that very reason [19,20]. In addition, we also examined the presence of F1 antigen on bacterial surface by performing immunofluorescence

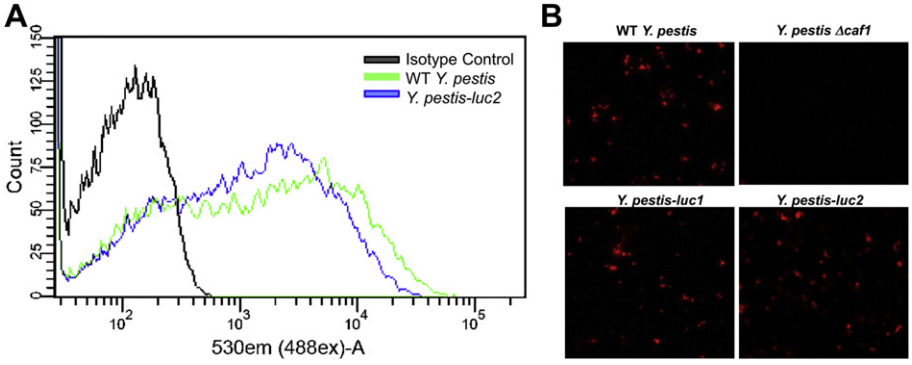

Fig. 1. A. Flow cytometric analysis of *Y. pestis-luc2* F1 antigen. The production of F1 antigen by the WT *Y. pestis* CO92 (green) and *Y. pestis-luc2* (blue) was evaluated by using specific antibodies to F1 or isotype-matched irrelevant antibodies (black), followed by detection with Alexa-488 labeled secondary antibodies. **B.** Immunofluorescence microscopy of fixed *Y. pestis* cells by using F1 primary antibodies and Alexa-594 conjugated secondary antibodies (magnification 400×).