

# METHOD FOR OPTICALLY MONITORING ANTHRAX INFECTION EVENTS IN REAL TIME USING LUCIFERASE TAGGED BACTERIA AND IN VIVO IMAGING TECHNIQUES

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## ABSTRACT

The recent exposures to *Bacillus anthracis* have highlighted certain issues that are unclear, especially the time frame for which spores could reside in the lungs, the dose, and their potential to subsequently cause a lethal outcome. It is important to find a technique that will allow for visual monitoring of the entire infection process of anthrax in real time to get a better understanding of the sequence of events and target tissues affected by this bacteria.

We have successfully transfected *B. anthracis* (Sterne) with the pXEN5 plasmid that contains the lux operon, kanamycin resistant colonies were selected that showed luminescent signals. In vitro assays on mouse macrophage cell line has been used to determine the infectivity of the transfectants. These luminescent transfectants will then be evaluated in an animal model. The utility of in vivo imaging, of a diseased animal has tremendous potential for testing therapeutic drugs or vaccines for their efficacy in an established animal model.

## 1. INTRODUCTION

The threat of terrorist action using biological warfare (BW), chemical or infectious agents has become a concern throughout the world. The agent that we propose to study is *B. anthracis* and design new technology to monitor the course of illness in real time and evaluate novel therapeutic strategies.

Recent events showed after inhalation of anthrax spores, the onset of the disease may occur within a day and death may follow rapidly in 5-7 days. Newly developed technology now makes it possible to track the location or effects of biological warfare (BW) agents in vivo in animals and to extend these studies over any time course desired while still keeping the animal alive

Host pathogen interactions triggered by an infection can be monitored in real time by a non-invasive imaging method. It is important to find a technique that will allow for visual monitoring of the entire infection process in real time to get a better understanding of the sequence of events and target tissues affected by this bacteria. We wanted to develop anthrax bacteria that will glow and produce light when injected into a live animal.

Methods for optically monitoring infectious events in vivo that involve an external light source may be limited by tissue-intrinsic background noise, even

with fluorescent markers. The technology using bioluminescence has a great advantage because of the absence of background light from mammalian cells. Imaging bioluminescence as an indicator of biological processes in animals can be used to address a number of issues in the study of pathogenesis, as well as efficacy for therapy and vaccine regimens.

This technology is different than measuring fluorescence since there is no need to have an external light source for excitation and then look for the emission. Use of luciferase reporter genes that are integrated into the genome and whose detectable signal is inextricably linked to the metabolic activity of the target cell population, produces a bioluminescent signal that can be captured by sensitive CCD cameras. Optimal reporter genes for in vivo cell trafficking studies should encode well-characterized gene products with deeply penetrating emission and a high signal-to-noise ratio.

Contag et al. (1995) reported the development of a method capable of detecting and monitoring bioluminescent bacteria within a living host by using an intensified charge-coupled device (ICCD) camera. Therefore, newly developed technology now makes it possible to track the location or effects of biological warfare (BW) agents in vivo in animals and to extend these studies over any time course desired while still keeping the animal alive. This is in vivid contrast with the early studies of localization whereby numerous animals would be sacrificed at each time point and laborious methods employed to recover a "labeled" BW agent.

Our approach was to optically monitor the infectious event of anthrax and evaluate efficacy of novel therapies in a live mouse model by using the new technology of bioluminescence and in vivo imaging. Host pathogen interactions triggered by an infection will be monitored in real time by a non-invasive imaging method. The utility of in vivo imaging, of a diseased animal has tremendous potential for testing therapeutic drugs (Francis et al, 2001) or vaccines for their efficacy in an established animal model. The second objective of the study is to design and test and evaluate novel therapies for anthrax using the in vivo imaging technique.

The present study deals with the design and development of luciferase tagged *Bacillus anthracis* (Sterne) to create luminescent bacteria for real time visualization and monitoring of the infection process by in vivo imaging in a rodent animal model.

# Report Documentation Page

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## 2. EXPERIMENTAL APPROACH

Bacterial strains, plasmid and culture media: The bacterial strain and plasmids used in this study are *Bacillus anthracis* (sterne) strain, and pXen5 plasmid was obtained from Xenogen, Inc.

The growth media used for cultivating *B. anthracis* were Brain Heart Infusion (BHI) from DIFCO, as well as BYGT. Sporulation was induced using Modified Schaeffer's Sporulation broth consisting of Difco Nutrient Broth, Potassium chloride, Magnesium Sulfate, Calcium Chloride, Ferrous sulfate monohydrate, Manganese chloride and Glucose as described in Leighton and Doi (1971).

Transformation and selection of *B. anthracis*: Plasmid DNA (pXen-5- Figure1) was isolated from *E. coli* using a plasmid isolation kit (Quiagen). The transformation method used in this experiment was adopted from Dunny et al. (1991) with minor modifications. Cells were electroporated using 2 $\mu$ g of plasmid pXen-5. The cells were first incubated for 90 min. at 30°C, after that were plated on selective BHI or LB media with antibiotics and incubated for 24 to 48 hr.

Screening for stable, highly bioluminescent

*B. anthracis* Sterne: Transformants of *B. anthracis* containing pXEN-5 were first patched onto LB-agar plates containing erythromycin (2 $\mu$ g/ml) and incubated overnight at 30°C. Erythromycin resistant colonies were then selected over different concentrations of LB-Kanamycin (up to 20 $\mu$ g/ml) at 42°C. The resulting colonies were then screened for bioluminescence signal using an ICCD camera and the brightest colonies were streaked onto LB agar plates containing kanamycin (1 $\mu$ g/ml). Each clone was then graded for its level of bioluminescence using an ICCD camera and Xenogen's LivingImage software (Xenogen Corporation, Alameda, Calif.)

## 3. RESULTS

Real time imaging is made possible by the use of bioluminescent bacteria and a powerful IVIS imager with a CCD camera. For the present study we transformed *B. anthracis* Sterne strain with the luciferase genes. The plasmid pXen-5 was used for transformation, which contains all the lux genes, erythromycin and kanamycin antibiotic resistance genes and the transposon that allows for chromosomal integration. Colonies resistant to kanamycin were selected for stable transfectants.

Selection of *B. anthracis* with chromosomal integration: Forty colonies were chosen from the erythromycin resistant bacteria and grown onto LB agar plates with different concentrations of kanamycin (Fig. 1). Only 12.5% colonies were able to survive at 20 $\mu$ g/ml of kanamycin. Although increasing the concentration of kanamycin in the medium gave rise to fewer

transformants, the proportion of colonies producing higher levels of bioluminescence was found to increase. This indicates that increasing the concentration of kanamycin in the medium results in the selection of integration sites with stronger promoters upstream of the luxABCDE *kmr* operon (Figure 2).

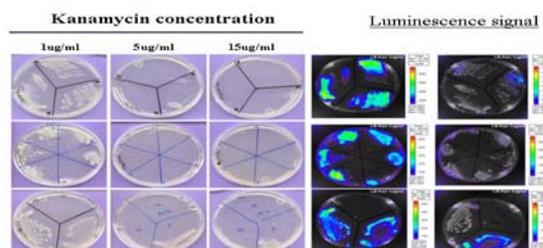


Figure 1. Kanamycin resistant colonies of *B. anthracis*. Colonies initially grown on LB erythromycin were re-plated onto LB plates with different concentrations of Kanamycin. Images taken with IVIS imager of Xenogen.

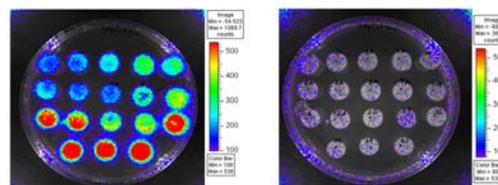


Figure 2. Luminescent colonies of *B. anthracis* grown in agar plates with kanamycin showing different luciferase activity as measured by the Xenogen's IVIS imager. Blue color shows less luminescent compared to red signal, which depict higher light signal.

## 4. CONCLUSION

The luciferase gene was inserted into the chromosome of *B. anthracis* Sterne strain and stable transfectants obtained. These luminescent anthrax bacteria will be used in an animal model for in vivo imaging to visualize the infection process in real time and test the efficacy of various novel therapeutic molecules.

## 5. REFERENCES

1. Contag, C. H., P. R. Contag, J. I. Mullins, S. D. Spilman, D. K. Stevenson, and D. A. Benaron. 1995. *Mol. Microbiol.* 18: 593-603.
2. Dunny, G., M., N. Lee, and D. J. LeBlanc. 1991. *Applied and Environmental Microbiology*, Apr. 1991: 1194-1201.
3. Francis, K. P., J. Yu, et al. 2001. *Infection and Immunity* 69, No. 5: 3350-3358.
4. Leighton, T. J, R.H. Doi. 1971. *Journal of Biological Chemistry* 246: 3189-95.