Salmonella *typhimurium infections* in balb/c mice: a comparison of tissue bioluminescence, tissue cultures and mice clinical scores

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SUMMARY

In response to systemic infection, mice usually present specific behaviors such as reduced activity and feeding, ruffled fur, hunched position, ataxia and tremor. We aimed to compare tissue bioluminescence, tissue cultures and clinical scores of BALB/c mice as potentially complementary outcome measures of *Salmonella* disease progression In Balb/c mice. The clinical status of the mice was assessed by visual examination for motility, ruffled fur, hunched position, feeding, ataxia and tremor. Patterns of bioluminescent light emission indicated the progression of infection from the abdominal region (initial site) to secondary tissue sites, which was indicative of systemic infection. As the severity and progression of infection increased, the bioluminescence signal became both more prominent and more anatomically disseminated. Bioluminescent Imaging (BLI) of *Salmonella* that have been genetically engineered to be bioluminescent is a new method that gives the opportunity to track *Salmonella* dissemination in mice. BLI is a helpful method to estimate tissue *Salmonella* concentration and may reduce the number of mice used in experiments, providing the opportunity to obtain serial assessments of disease progression in a single mouse subject. Clinical scores helped us to assess the clinical status of BALB/c mice in systemic *Salmonella* infections.

KEY WORDS: Salmonella, Diagnosis, Tissue biotechonology

Received July 13, 2011 *Accepted* October 13, 2011

INTRODUCTION

Mice experiments play an important role in all aspects of medical research. Systemic infection models in mice have been widely used in the last 20 years. Mice are especially chosen for experimental studies in infection models because of their anatomic similarities with humans.
Murine salmonellosis is a widely used experimental model for acute systemic salmonellosis in humans because the invasive disease caused by *Salmonella typhimurium* in mice resembles the acute phase of human typhoid fever caused by *Salmonella typhi* (Maw *et al.,* 1968).
Salmonella species are gram-negative, facultative intracellular bacteria that are distributed globally. *Salmonella enterica* serovars *typhimurium*, *typhi* and *enteriditis* cause the vast majority of human infections worldwide (Wickham *et al.,* 2007).
BALB/c mice are widely used to study the pathogenesis of serotype *typhimurium* infections. Some of the behavioral responses of mice to systemic bacterial infections have been described in the literature (Burns-Guydish *et al.,* 2005).
Salmonella typhimurium has emerged as a model pathogen bacteria. Using bioluminescent imaging, investigators may estimate local tissue S*almonella* concentrations (Burns-Guydish *et al.,* 2005).

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MATERIALS AND METHODS

This study was undertaken at the McDonald Pediatric Research Center, Washington University in St.Louis, MO from December 2005 to December 2006.

Mice

A hundred six-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, Maine, USA) and 100 control six-week-old female BALB/c mice were maintained by the Division of Comparative Medicine at Washington University School of Medicine in accordance with all applicable federal guidelines with protocols approved by the Washington University animal studies committee. Prior to peroral inoculation, the mice were deprived of food overnight and of water for 3 hours. They were then fed with 50 µl of log-growth-phase bacteria suspended in Luria broth (LB). The actual dosage was confirmed by plating serial dilutions of the inoculum. The mice were housed in cages and fed with sterilized water and food *ad libitum*.

Clinical assessments and weight measurements were performed daily to track the condition of the mice. The average weight of the study group was 23.64±1.61 grams and the control group was 24.38±1.63 grams. Mice were euthanized when systemic dissemination of infection was apparent (displayed overt signs of systemic infection, such as ruffled fur, lethargy, hunched posture, ataxia and tremor) or had high bioluminescent signals from tissues. To determine the number of bacteria in host tissues, bioluminescent imaging (BLI) and tissue cultures were used. After the mice were sacrificed their livers were extracted, homogenized in sterile phosphate buffer solution (PBS) and plated for colony counts.

Bacterial strains and growth conditions

Bacterial strains were derived from the wild-type S*almonella enterica serovar typhimurium*. We created the constitutively bioluminescent *Salmonella* strain, SB300A1 FL6 via chromosomal integration of the P. luminescens luxCDABE operon, using a mini-Tn5 luxCDABE-Kan system (Winson *et al.,* 1998). Bacteria were grown in LB. *Salmonella* strains were cultured at 37°C overnight without agitation in LB. The following day, bacteria were diluted 1:30 in LB and agitated for about 3 hours at 37°C until the optical density at 600 nm reached 0.3. The actual number of bacteria present was determined by viable-cell counting. Wild-type bacteria were recovered from the liver of BALB/c mice. Aliquots (100 l) of the tissue suspensions were serially diluted in PBS to a maximum of 10^6 fold dilution and then were plated on duplicate MacConkey agar plates. Plates were incubated overnight at 37°C, luminescent colonies were counted, and CFU were obtained.

Noninvasive detection of *Salmonella* bioluminescence

BLI is a noninvasive and versatile in vivo imaging modality. BLI was performed using an imaging system equipped with a cooled CCD camera. Light transmission through tissues depends on the wavelength of luciferase emission and depth of tissue.

We gavaged 6-week-old BALB/c mice with wild-type bioluminescent *Salmonella typhimurium* with a dose of 10^7 CFU/ml and progression of infection was monitored by BLI. The control group was gavaged with saline. Images were taken by CCD camera. Mice were anesthetized with thiopentol, an inhalational anesthetic and then placed in the imaging system (IVIS 1000, Xenogen, Alameda, CA, USA) (Figure 1). Bioluminescent images were captured with a

FIGURE 1 - *Live mice and extracted liver were imaged with a CCD camera mounted on a black box (Xenogen IVIS System). Acquired data can be quantified with imaging software.*

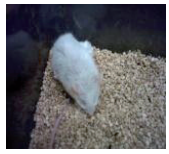
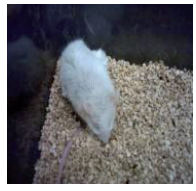
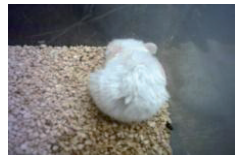
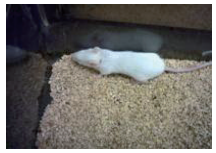
Clinical Findings		**Clinical Scores**	
	0	**1**	**2**
Activity Level	No activity 	Reduced activity 	Normal 
Feeding	No feeding 	Decreased feeding 	
Appearance of Fur	Overt ruffled 	ruffled 	
Hunched position	Complete round 	Partial round 	
Ataxia, tremor	Overt 	Mild 	

FIGURE 2 - *Clinical findings of BALB/c mice in response to systemic Salmonella typhimurium infection.*

cooled CCD camera 5 days after the mice were infected.
5-minute images of light transmitted through the animal's tissues were taken in the dark. After photon collection, a pseudocolor representation of light intensity (red, most intense; blue, least intense) was superimposed over the grayscale body surface reference image signals were measured as the photon flux range between 200-4500 (photons/second).

Clinical evaluation of mice
The clinical evaluation was made according to theclinical response of BALB/c mice to systemic *Salmonella* infection. We developed a clinical scoring system. The components of this system were: activity of mice, feeding, appearance of fur, hunched position, ataxia and tremor (Figure 2). The points used to assess the clinical status were 0, 1 and 2. The average of three measures was taken. If the average total score was equal to or below 3, the mice were accepted as severely sick. If the total score was between 4 and 7, the mice were accepted as moderately sick, if the total score was equal or over 8, the mice were accepted as healthy.

Statistical analysis
The Pearson analyses test was applied to the results. Correlation is significant at 0.01 level (2-tailed).

RESULTS

The clinical status of the mice was assessed by visual examination for motility, ruffled fur, hunched position, feeding, ataxia and tremor. We compared tissue bioluminescence, clinical scores and tissue cultures of BALB/c mice. The CS for wild-type *Salmonella* were equal to or below 7 and equal to or over 8 for the control group. Patterns of bioluminescent light emission indicated the progression of infection from the abdominal region (initial site) to secondary tissue sites, which was indicative of systemic infection. As the severity and progression of infection increased, the bioluminescence signal became both more prominent and more anatomically disseminated.
BLI signal detection was sensitive enough to detect as few as 3 log10 CFU/ml *Salmonella* (obtained in tissue cultures) detected by BLI both in vivo and ex vivo (Figure 3).
The Pearson analyses test was applied to the results. The CS correlated negatively with in vivo and in vitro tissue bioluminescence and tissue cultures for wild-type *Salmonella typhimurium* (r=-0.686, r=-0.659 and -0.807 respectively). The p value was below 0.0001. There was more than 7 log10 CFU wild-type *Salmonella* growth in tissue cultures in the study group (Table 1, Figures 3, 4).

TABLE I - *Correlations and p values.*

		Mice Bioluinescence	*Liver Bioluminescence*	*Tissue Cultures*	*Clinical Scores*
Mice Bioluinescence	Pearson Correlation	1,000	,630	,792	-,686
	p		,0001	,0001	,0001
Liver Bioluminescence	Pearson Correlation	,630	1,000	,770	-,659
	p		,0001	,0001	,0001
Tissue Cultures	Pearson Correlation	,792	,770	1,000	-,807
	p		,0001	,0001	,0001
Clinical Scores	Pearson Correlation	-,686	-,659	-,807	1,000
	p		,0001	,0001	,0001

**Correlation is significant at the 0.01 level (2-tailed).

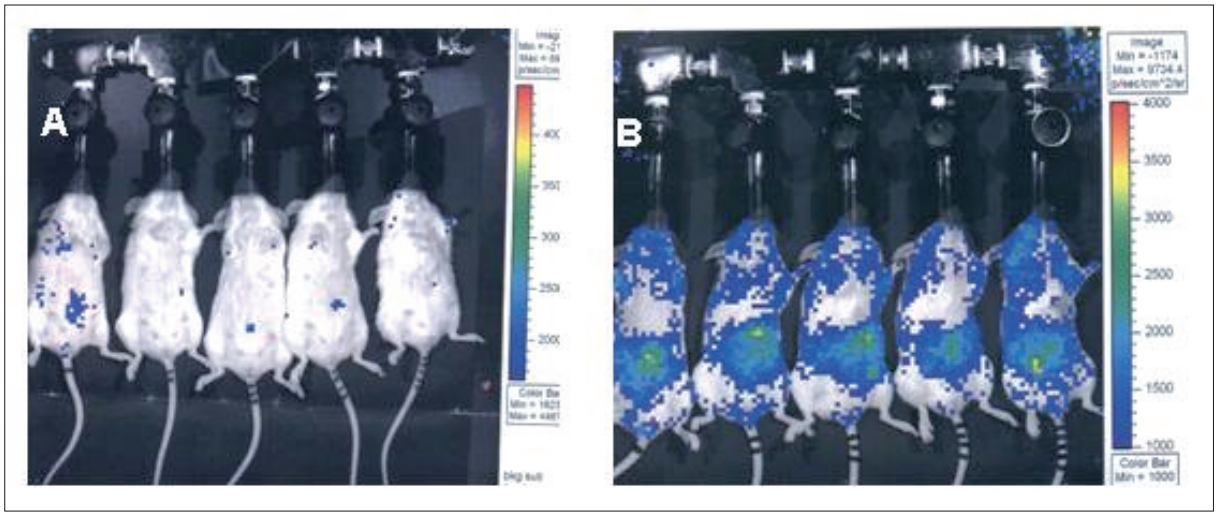


FIGURE 3 - *Mice Bioluminescence in study and control group (r=-6,86), p<0.0001. A) 6-wk-old BALB/c control mice given saline by orogastric gavage. B) 6-wk-old BALB/c mice infected with 107 CFU/ml wild type bioluminescent Salmonella by orogastric gavage. Biouminescent images were taken by CCD camera 5 days after the mice were infected. Light intensity is represented by a color scale in counts. Rainbow scale represents photon counts. Patterns of light emission indicate the progression of infection from the abdominal region (initial site) to secondary tissue sites, which is indicative of systemic infection.*

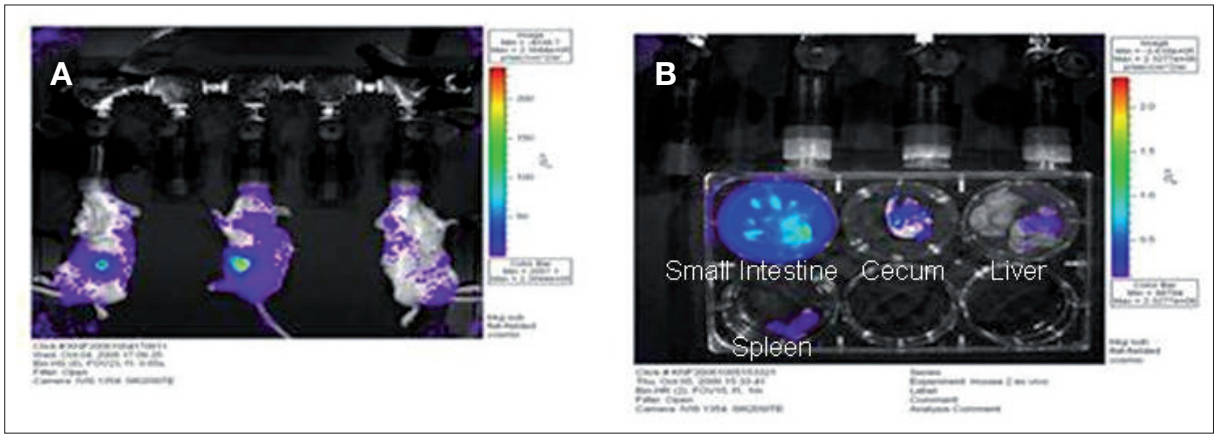


FIGURE 4 - *A) In vivo BLI. B) Ex vivo BLI. As low as 3 log10 CFU/ml Salmonella (obtained in tissue cultures) could be detected by BLI both in vivo and ex vivo.*

DISCUSSION

Acute gastroenteritis caused by *Salmonella typhimurium* is an important disease which is life-threatening to immunocompromised individuals in the West, and fatal to children and older people in developing countries. An estimated 1.3 billion cases/year are responsible for 3 million deaths worldwide.

Studying a natural infection in mice which develops signs of disease similar to those observed in humans is essential (Santos *et al.,* 2001).

Tissue culture as an experimental system provides investigators with the ability to study the interactions of a single pathogenic organism with a single host-cell type in a more controlled enviroment (Hurley *et al.,* 2003).

Salmonella invasion of host cells is mediated in part by a set of genes known as the *Salmonella* Pathogenicity Island I (SPI-1). Following infec-

tion with *Salmonella* susceptible mice develop a systemic disease characterized by rapid bacterial multiplication in the liver at a net growth rate of 0.5-1.5 log/day which results in hepatomegaly (Hapfelmeier *et al.,* 2005; Contag *et al.,* 1998 ;Hormaeche 1980). In mice, invasion of the gut epithelium is followed by bacteraemia and proliferation inside deeper target tissues such as the liver. Inside phagocytic cells, *Salmonella* are capable of escaping the host response that would normally kill most other infectious agents. Gross pathology of the intestine commonly reveals enlarged peyer's patches and a thickening of the ileal mucosa (Santos *et al.,* 2001).

The gram-negative bacterium S*almonella enterica* serovar typhimurium causes enterocolitis in humans and a systemic typhoid-like disease in mice. An initial step in virulence of S*almonella* in mice involves attachment to the phagocyte surface before the bacteria are ingested and transported by the lymphatic system to the liver and spleen (Buchmeier *et al.,* 1991).

Wild type S*almonella typhimurium* carrying a mutation in S*almonella* pathogenicity island genes were used in this study.

Studies on the pathogenesis of S*almonella* enterica serovar typhimurium infections in mice have revealed two prominent virulence characteristics: the invasion of the nonphagocytic cells to penetrate the intestinal epithelium, and the proliferation within host phagocytic cells to cause a systemic spread and the colonization of host organs.

Following oral infection of mice, bacteria adhere to and invade cells of the intestinal epithelium, survive in blood, proliferate in macrophages, and access systemic sites through the lymphatic and blood circulation systems (Carter *et al.,* 1974; Finlay 1994).

Bioluminescence imaging (BLI) technology can be applied to many aspects of cell biology ranging from the analysis of specific markers in cells and tissues to the biological actions and distributions of fluorescent proteins or particles in living cells (Buda 2005).

Bioluminescence imaging (BLI) is a noninvasive and versatile in vivo imaging modality. Light transmission through tissues depends on the wavelength of luciferase emission and depth of tissue. Thus, photons that emit from superficial and translucent tissues, such as lymph nodes, bones or skin, will transmit with less scattering and absorption than from opaque organs such as liver (Burns-Guydish *et al.,* 2005). BLI is a technology to monitor the progression of infection in mice. The advantage of BLI is that the technology cannot only reveal spatiotemporal patterns of infection but also guide the timing of ex vivo assays and the selection of tissues for labor-intensive and time-consuming assays.

The use of mouse models to understand bacterial infections and for testing the efficacy of antibiotics has been well established, and the use of BLI to accelerate the analyses of these models has been reported (Contag *et al.,* 1998).

The components of the CS were reduced activity and feeding, hunched posture, ruffled fur, ataxia and tremor. There was a negative correlation between the CS, tissue cultures and in-vivo, ex-vivo tissue bioluminescence. CS may be a prototype method to assess laboratory mice's clinical condition in systemic infection models and may be used as a helpful method to assess the clinical condition of BALB/c mice during systemic *Salmonella typhimurium* infections. The bioluminescent imaging method may help to reduce the number of mice used in experiments compared with conventional blind LD50 studies and provide opportunity to use the same mice throughout the study. BLI may help to predict the number of bacteria (CFU) in murine tissues and confirm and compare these predictions with traditional CFU determinations.

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