ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* are important opportunistic bacteria responsible for serious hospital infections. These pathogens have become problems worldwide because of their high prevalence and resistance to a wide range of antibiotics. In this study, lethal and sublethal doses were estimated by intraperitoneal (IP) infection with *A. baumannii* and MRSA in C57-BL6 and Balb/c mice, respectively. Twenty-four hours post-infection, animals belonging to the groups that received doses of *A. baumannii* ATCC19606 greater than $2 \times 10^5$ CFU/mL were not able to survive; this was considered the lethal dose. However, some animals that received doses below $2 \times 10^4$ CFU/mL survived, allowing the LD$_{50}$ for this bacterium to be established at approximately $1 \times 10^5$ CFU/mL. Meanwhile, groups that were infected with MRSA were able to survive at doses below $1.2 \times 10^6$ CFU/mL, which was found to be the LD$_{50}$ in this trial. The Three Rs of humane animal experimentation were observed, allowing the reliable and rapid estimation of the lethal and sublethal doses in two models, using different mouse lineages and bacterial strains and employing four animals per group instead of 10, as recommended in the literature. We suggest that the same protocol could be used with other bacteria to determine lethal and sublethal doses, thus vaccine assays and other antibacterial strategies could be evaluated more quickly while using fewer animals.

KEYWORDS: *Acinetobacter baumannii*; MRSA; lethal dose; murine model; nosocomial infection
INTRODUCTION

*Acinetobacter baumannii* and Methicillin-resistant *Staphylococcus aureus* (MRSA) are important opportunistic bacteria that cause pneumonia, urinary tract and skin tissue infections, and septicemia in immuno-compromised patients. Infections caused by these pathogens frequently occur in patients with large burns, patients in intensive care under mechanical ventilation, and patients submitted to invasive procedures (Perez et al. 2007; Towner, 2009).

The emergence and propagation of multidrug-resistant bacteria has become common in the world, and there are limited therapeutic options, requiring the identification of alternatives for treating infections caused by these pathogens (Jones et al. 2007; Lin et al. 2008). In this respect, vaccines and passive immunotherapies are interesting alternatives to the current antibiotic therapies used to treat infections caused by *Acinetobacter spp.* and MRSA.

As with most bacterial diseases, experimentation in animals is necessary to evaluate and characterize elicited immune responses and protection after bacterial challenge assays. The humanitarian principle of animal experimentation is defined as the principle of the Three Rs (replacement, reduction, and refinement). Replacement means that whenever possible, unanimated materials (without sensitivity), such as tissue cultures or computer models, should be used instead of living animals. Reduction indicates that the minimum number of animals necessary to elucidate meaningful results from the experiment should be used. Refinement requires minimizing invasive techniques and handling animals only by trained personnel (Andrade et al. 2006).

Therefore, the objective of this study was to establish the lethal doses (LDs) and sublethal doses of *A. baumannii* and MRSA bacteria in murine models, adapted from the Reed and Muench method (Reed and Muench 1938) in a fast and reliable way while using fewer animals, meeting the principle of the Three Rs. These results could be important for estimating the bacterial load to be used in animal challenge assays during the development of vaccines, immunotherapeutics, or other antibacterial approaches.

MATERIALS AND METHODS

Animals

Adult female Balb/c and C57 Black mice (8 weeks, 18 g) provided by the Animal Breeding Center (CECAL) of FIOCRUZ were used. All procedures were approved by the Ethics Committee on Animal Research (CEUA), FIOCRUZ (LW 71/12 and LW 7/11). Because LD experiments tend to induce suffering in animal methods intended to reduce any kind of distress were necessary. The animals were closely accompanied and monitored to check for signs of suffering or morbidity. Abnormal symptoms in the animals were considered as endpoints (Stokes 2002), leading to immediate euthanasia, according to humane endpoints guidelines (CCAC 1998; ARAC 2013; National Research Council 2011).

Bacterial strains and growth conditions

Overnight cultures of *A. baumannii* strain ATCC 19606 and MRSA (COL strain) were diluted 1:100 with fresh medium, and the bacteria grew to the exponential phase. The cultures were diluted to the appropriate bacterial concentration using phosphate buffered saline (PBS) 1x. Bacterial concentrations in the inoculum were determined by plating 10-fold dilutions on LB agar followed by dilution of 500 µL in sterile PBS 1x containing 2.5% mucin (Sigma M1778) administered intraperitoneally (McConnell et al. 2011).
Estimation of lethal and sublethal doses

Lethal and sublethal doses were estimated by intraperitoneal infection of four groups (n=4) of C57-Black mice with *A. baumannii* ATCC 19606 and infection of four groups (n=4) of Balb/C mice with MRSA using the modified Reed-Muench method (McConnell et al. 2013). The groups infected with *A. baumannii* received the following doses respectively: 2.0x10^6, 2.0x10^5, 2.0x10^4, and 2.0x10^3 CFU. The groups infected with MRSA received 1.2x10^7, 1.2x10^6, 1.2x10^5, and 1.2x10^4 CFU. Mice remained under observation for seven days. After this period, the lethal and sublethal doses were estimated as the number of animals that survived after infection (McConnell et al. 2011).

![Graph showing survival rates](image)

**Figure 1:** Survival mice after intraperitoneal infection of the indicated inoculum (2.0x10^3 to 2.0x10^6 CFU) of the *A. baumannii* ATCC19606 strain (n=4 mice/group).

RESULTS & DISCUSSION

Twenty-four hours after infection with *A. baumannii*, all mice that received 2.0x10^5 or 2.0x10^6 CFU did not survive, while those who received 2.0x10^3 CFU did survived the infection (Fig. 1). In the group infected with 2.0x10^4 CFU, one mouse died in the first 24 hours while the other mice survived. Thus, the LD of *A. baumannii* was estimated to be approximately 1.0x10^5 CFU. The lowest dose at which all mice survived was 2.0x10^3 CFU, which under these conditions, was considered the sublethal dose.

Twenty-four hours after infection with MRSA, all animals that received 1.2x10^7 CFU did not survive, whereas those that received 1.2x10^4 or 1.2x10^5 CFU remained alive (Fig. 1) after infection. In the group infected with 1.2x10^6 CFU, two mice died in the first 24 hours while the other mice survived. Thus, the LD and LD_50 of MRSA were estimated to be approximately 1.2x10^7 and 1.2x10^6 CFU, respectively. The lowest dose at which all mice survived was 1.2x10^5 CFU; therefore, under these conditions, this can be considered the sublethal dose. These results are shown in Fig. 2.
These results were reproducible and allowed determination of the lethal and sublethal doses of these bacteria to evaluate immune protection against these pathogens. Mice infected at the established sublethal dose were used for recovery of bacteria from tissues (kidneys, spleen) for both models, which is important for estimating systemic protection from any treatment (vaccine, antibody-based, or A major challenge in the use of animal models to characterize infectious pathologies is accurately mimicking human diseases. Although it is unrealistic to expect that infection of laboratory animals, which in many cases are inbred strains, can completely reproduce the human disease process, in this case, efforts should be made to simulate these conditions as closely as possible (McConnell 2013). Mouse infection models in wild type animals usually need to use large amounts of inoculating bacteria, compared to those used in human infections. To overcome this limitation, mucin was used at a final concentration of 2.5%, administered in the bacterial inoculum. This approach allowed the reduction, by about 1000-fold, of the bacterial load in the inoculum with the same bacteria recovered from kidneys (data not shown).

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![Figure 2](image-url): Survival mice after intraperitoneal infection of the indicated inoculum (2.0x10^3 to 2.0x10^6 CFU) of the MRSA COL strain (n=4 mice/group).
In closing, we suggest that the present protocol could be applied in other bacterial infection models to determine lethal and sublethal doses using fewer animals. This adapted protocol proved to be simple, easy to carry out, and reproducible, allowing results to be obtained in few days. These data provide important information used to determine the bacterial concentration necessary to challenge the animals during the development of future vaccines and antibody-based therapies against bacterial diseases.

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REFERENCES


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