ABSTRACT - Background: Animal models are useful to evaluate the efficacy of antimicrobials in experimental sepsis. Aim: To elucidate the steps of producing an experimental model for the treatment of extended-spectrum beta-lactamase (ESBL)-producing Klebsiella pneumoniae sepsis. Methods: Several ESBL inoculums ranging from 1.5x10^9 colony-forming units per milliliter (CFU/mL) to 2.0x10^10 CFU/mL were administered by peritoneal injection in adult Wistar rats. Outcomes and microbiological data of quantitative peritoneal and blood cultures were observed in untreated animals. Animals which received 2.0x10^10 CFU/mL inculcums were treated with single meropenem dose (30mg/kg) after one hour and those which received 1.0x10^9 CFU/mL inoculums were treated immediately with three doses of meropenem 50 mg/kg. Outcomes were observed for 24 hours after inoculation. Results: Solutions with 1.5x10^9 and 6.0x10^9 CFU/mL were not lethal within 24 hours. Inoculums of 1.0x10^10 CFU/mL were lethal in 80% and solutions with 2.0x10^10 CFU/mL were lethal in 100% of animals. ESBL lethal sepsis (1.0x10^10 CFU/mL) was treated immediately with 50 mg/kg of meropenem every eight hours for 24 hours and presented 40% mortality compared with 80% mortality of the control group (p=0.033). Quantitative cultures of peritoneal fluid present 10^5 CFU/mL or less for treated animals compared to more than 10^8 for untreated animals (p=0.001). Conclusion: Inoculums of 1.0x10^10 CFU/mL achieved the best results to study a model of lethal sepsis and this model of treatment of carbapenem-susceptible Enterobacteriaceae can serve as control to further evaluation of treatment of carbapenemase-producing Enterobacteriaceae models.

INTRODUCTION

The incidence of carbapenemase-producing Enterobacteriaceae has increased and the ideal treatment has not been established. Some retrospective studies suggest an association of different drugs to improve outcomes1,12. Antibiotic therapy to specific bacteria can be evaluated based on clinical outcomes, mortality, time to death and rate of microbiological cure. Considering these findings, an experimental model might be helpful to evaluate the combination of different drugs to treat carbapenemase-producing Enterobacteriaceae until clinical studies confirm the benefits of this approach. To study animal models on carbapenemase-producing Enterobacteriaceae, a treatment control of extended-spectrum betalactamase (ESBL)-producing Klebsiella pneumoniae must be validated.

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model of ESBL-producing *Klebsiella pneumoniae* treatment with meropenem. Models of peritonitis in rats evaluated inoculums ranging from $10^8$ to $10^{10}$ colony-forming unit per milliliter (CFU/mL) of *E. coli*. Lethal sepsis was observed at higher inoculum concentrations ($10^9$ to $10^{10}$ CFU/mL). Non-letal models were done with $10^7$ to $10^8$ CFU/mL inoculums. *Klebsiella pneumoniae* was evaluated in peritonitis of neutropenic mice ($3 \times 10^7$ CFU/mL), thigh infection in neutropenic rats ($10^7$ to $10^8$ CFU/mL)\(^a\) and pneumonia models in rats ($10^8$ to $10^{10}$ CFU/mL)\(^b\). *Enterobacter* spp. was also evaluated in a pneumonia model of $10^6$ CFU/mL.\(^d\)

*Klebsiella pneumoniae* inoculum concentrations must be standardized to determine a sepsis model that might be able to evaluate the efficacy of antimicrobial therapy in preventing lethality serving as a control for treatment of carbapenem-resistant *Klebsiella pneumoniae*. This study aims to describe the more adequate inoculum concentration to induce lethal but treatable sepsis. Timing and dose of antimicrobial therapy for ESBL peritoneal sepsis induced in non-neutropenic rats were evaluated.

**METHODS**

**Animals**

The experiment was performed with adult (20–24 week old) male and female Wistar rats weighting 200-340 g. Animals were maintained under artificial day-night cycles, adequate temperature (22–24 °C) and humidity. The rats received a standard diet and water ad libitum. Animals were allowed to adapt to laboratory conditions for two days. The animal research ethics committee of the Universidade Estadual de Ponta Grossa approved the study. Fifty rats were included in the phases of this experiment.

**Bacterial strain, inoculum production and sepsis induction**

ESBL-producing strain (ATCC 700603) was inoculated into Mueller-Hinton broth and incubated at 37°C for 24 h. Colonies were suspended in sterile isotonic saline solution to form the inoculums.

To accurately measure the inoculum a densimeter (Densimat Biornerieux\(^c\)) capable of measuring densities of 0.5 to 7.5 McFarland was used to evaluate the inoculums of $1.5 \times 10^9$ CFU/mL which was obtained at 5 McFarland. To accurately measure more concentrated inoculums, spectrophotometry (Lambda 25 UV/Vis Spectrophotometer Perkin Elmer\(^e\)) was performed at optic density of 625 nm. Inoculums with $1.5 \times 10^{10}$ and $2.0 \times 10^{10}$ CFU/mL corresponded to solutions of barium chloride and sulfuric acid of 50 and 67 McFarland standards and the absorbencies of these solutions were 2.343 and 2.764 respectively. According to Beer-Lambert law, absorbencies over 0.890 are not accurate for measuring microorganism counts. After 1:20 dilution, inoculums with $1.5 \times 10^{10}$ and $2.0 \times 10^{10}$ CFU/mL presented absorbencies of 0.543 and 0.633. Inoculums of $6.0 \times 10^7$ CFU/mL and $1 \times 10^7$ were obtained by injection of 0.4 mL and 0.6 mL of $1.5 \times 10^7$ CFU/mL solution.

All inoculums were incubated at Mueller-Hinton and CFU were counted eight hours latter to confirm the concentration before animal injection.

Sepsis was induced by intra-peritoneal injection of the inoculum using a 26 gauge needle in the lower right abdomen. All the procedure was performed under aseptic conditions. Inoculum lethality was defined by injection of $1.5 \times 10^8$ CFU/mL solution in six animals, $6.0 \times 10^7$ CFU/mL in five, $1.0 \times 10^10$ CFU/mL in ten animals and $2.0 \times 10^10$ CFU/mL in ten animals.

**Antimicrobial therapy**

Two groups of ESBL lethal sepsis were treated with meropenem (Astra-Zeneca\(^c\)). Twelve rats were inoculated with $2.0 \times 10^{10}$ CFU/mL and six of them were treated with one dose of meropenem 30 mg/kg after one hour of inoculation. Twenty animals were inoculated with $1.0 \times 10^{10}$ CFU/mL and ten were treated immediately with 50 mg/kg of meropenem every eight hours for 24 hours. Homogeneous distribution of animals by weight and sex were done in treated and untreated groups.

**Outcome evaluation**

The rate of lethality, length of survival, blood cultures positivity and quantitative peritoneal fluid and peritoneal tissue cultures were evaluated. Cultures were obtained aseptically.

Animals not presenting lethal sepsis after 24 h suffered euthanasia with lethal doses of xylazine and quetamine. Blood cultures (0.5-1.0 mL) were collected through cardiac puncture after death or euthanasia and incubated in brain heart infusion broth. Peritoneal fluid was obtained after laparotomy and injection of 5 mL of isotonic saline and aspiration. One microliter of this fluid was cultured in McConkey agar. Quantitative cultures were performed after 1:100 dilutions of the peritoneal solution in isotonic saline and incubation of 1μL in McConkey agar.

**Statistical analysis**

Continuous data were expressed as mean±standard deviation (SD), frequencies were expressed as percentages. Dichotomous variables were compared using Mann-Whitney test. Kruskal-Wallis test was used to evaluated hours of survival of the four untreated groups. Significance level was set at 0.05. All data were stored using the software Excel (Microsoft, New York, USA) and statistical analysis was performed using the software SPSS 16 (SPSS, Chicago, USA). Graphics and statistical analysis by Mann-Whitney were performed with GraphPad Prism 5.0 (GraphPad, La Jolla, USA).

**RESULTS**

ESBL solutions ranging from $1.5 \times 10^9$ to $2.0 \times 10^{10}$ CFU/mL were evaluated. Solutions with $1.5 \times 10^9$ CFU/mL were not lethal in 100% of animals. Inoculums of $6.0 \times 10^9$ and $1.0 \times 10^{10}$ CFU/mL were lethal in 80% rats. Solutions with $2.0 \times 10^{10}$ CFU/mL were lethal in 100% of animals (Figure 1). ESBL lethal sepsis ($2.0 \times 10^{10}$CFU/mL) was treated with meropenem one dose of 30 mg/kg after one hour of inoculation with no improvement on mortality. Other group of ESBL lethal sepsis ($1.0 \times 10^{10}$ CFU/mL) was treated immediately with 50 mg/kg of meropenem every eight hours for 24 hours presented 40% mortality, significantly lower than 80% mortality of the control group (p=0.042, Figure 2). Quantitative cultures of peritoneal fluid presented $10^9$ CFU/mL or less for treated animals compared to more than $10^9$ for untreated animals (p=0.001, Figure 3).

**DISCUSSION**

Previous studies described models of peritoneal inoculums of *E. coli* between $10^9$ and $10^{10}$ CFU/mL to achieve lethal and non-lethal sepsis\(^f\).\(^{1,5,11}\) Recent models of neutropic rats with thigh infection are performed with lower concentrated inoculums and usually do not evaluate mortality, only microbiologic efficacy.\(^f\) Models with *Klebsiella* spp. are less frequent and must be validated. Here is described the standardization of a lethal model of peritonitis by ESBL-producing *K. pneumoniae* passible of treatment in non immunosuppressed rats.
Solutions of 10^8 and 10^9 UFC/mL cause non-lethal sepsis in immunocompetent rats, which are useful to stratify antimicrobial dosing and compare antimicrobial efficacy on microbiological results, but are not ideal to compare antimicrobial efficacy on clinical outcomes. Was observed that a single antimicrobial dose after inoculation might not be adequate to differentiate treated and untreated animals. Furthermore, was also observed that inoculation of 2.0x10^10 CFU/mL with no immediate treatment, cause lethal sepsis that may not be adequate to evaluate antimicrobial efficacy on survival, since most animals may die in spite of treatment.

Inoculums of more than 1.0x10^10 and less than 2.0x10^10 colony-forming units per milliliter, accurately measured by spectrophotometry, produce lethal sepsis. Immediate treatment after inoculation, administered for 24 hours permits to compared outcomes and microbiological samples of treated and untreated animals. The immediate antimicrobial infusion was based on previous studies. Was thought that immediate infusion of antibiotic could reduce the bacterial burden, but both groups had positive cultures in the end of the experiment.

This study validates an animal model of sepsis which induced lethal peritonitis in the control group between six and 24 hours and the treated group had cultures with significantly fewer microorganisms. Data from quantitative cultures, length of survival and mortality can serve as a control to evaluate the treatment of carbapenemase-producing Enterobacteriaceae models.

**CONCLUSION**

Inoculums of 1.0x10^10 CFU/mL achieved the best results to study a model of lethal sepsis and this model of treatment of carbapenem-susceptible Enterobacteriaceae can serve as control to further evaluation of treatment of carbapenemase-producing Enterobacteriaceae models.

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