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Two bacterial infection models in tree shrew for evaluating the efficacy of antimicrobial agents

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Abstract: Animal models are essential for the development of new anti-infectious drugs. Although some bacterial infection models have been established in rodents, small primate models are rare. Here, we report on two bacterial infection models established in tree shrew (*Tupaia belangeri chinensis*). A burnt skin infection model was induced by dropping 5×10^6 CFU of *Staphylococcus aureus* on the surface of a wound after a third degree burn. This dose of *S. aureus* caused persistent infection for 7 days and obvious inflammatory response was observed 4 days after inoculation. A Dacron graft infection model, 2×10^6 CFU of *Pseudomonas aeruginosa* also caused persistent infection for 6 days, with large amounts of pus observed 3 days after inoculation. These models were used to evaluate the efficacy of levofloxacin (LEV) and cefoperazone (CPZ), which reduced the viable bacteria in skin to $4\log_{10}$ and $5\log_{10}$ CFU/100 mg tissue, respectively. The number of bacteria in graft was significantly reduced by $4\log_{10}$ CFU/mL treatment compared to the untreated group ($P<0.05$). These results suggest that two bacterial infection models were successfully established in tree shrew using *P. aeruginosa* and *S. aureus*. In addition, tree shrew was susceptible to *P. aeruginosa* and *S. aureus*, thus making it an ideal bacterial infection animal model for the evaluation of new antimicrobials.

Key words: Tree shrew; Novel antimicrobials; Graft infection; *Staphylococcus aureus*; *Pseudomonas aeruginosa*

树鼩细菌感染模型的建立及抗菌药物的治疗效果评价

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摘要：在抗微生物感染药物开发过程中, 动物模型是必不可少的。虽然目前已经用啮齿类动物建立了一些细菌感染动物模型, 但在小型灵长类动物中还很少见。这里首次报道两个树鼩细菌感染动物模型。第一种是在三度烫伤后的皮肤表面接种 5×10^6 CFU 的金黄色葡萄球菌构建的皮肤烫伤感染模型。这个数量的金黄色葡萄球菌可以造成 7 d 持续性感染, 并且在第 4 天可以看到明显的炎症反应。第二种是用绿脓杆菌构建的涤纶补片感染模型, 接种 2×10^6 CFU 的绿脓杆菌同样可以引起持续 6 d 感染, 并在第三天在伤口处观察到大量的脓液。进一步用这两种模型评价头孢哌酮钠和左氧氟沙星的治疗效果。左氧氟沙星和头孢哌酮钠在皮肤烫伤感染模型中能分别将 100 mg 皮肤组织中的细菌降低到 $4\log_{10}$ 和 $5\log_{10}$ CFU, 并且在涤纶补片植入感染模型中这两种抗生素都能显著地将感染的细菌降低了 $4\log_{10}$ CFU ($P<0.05$)。结果表明用金黄色葡萄球菌和绿脓杆菌成功构建了两个细菌感染的树鼩模型。此外, 树鼩对金黄色葡萄球菌和绿脓杆菌很敏感, 适合用于构建细菌感染动物模型和评价新的抗细菌感染药物的效果。

关键词：树鼩; 新型抗菌药物; 移植物感染; 金黄色葡萄球菌; 绿脓杆菌
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Bacterial infection is a serious disease affecting the health of humans. Traditional antibiotics have been widely applied in clinics since the first antibiotic, penicillin, was discovered, which has resulted in the emergence of many antibiotic-resistant strains all over the world (Fischbach & Walsh, 2009; Givskov & Hentzer, 2003; McPhee & Hancock, 2005; Oyston et al, 2009; Zasloff, 2002). More powerful antimicrobials are urgently needed to conquer infections induced by drug-resistant bacteria. Testing the therapeutic potential of new antimicrobial drugs in animal models is a very important stage of that process (Craig, 1993). To date, bacterial infection models have been established in mice, rats, and rabbits, but no models have been developed for small primate species (McCormick et al, 2008; Retsema et al, 1993; Russo et al, 2008). The mechanisms of infectious disease in non-primates are not similar with humans and although some antimicrobials have been found useful in non-primate animal models, they lost their activity during clinical trials(Druilhe et al, 2002). Murine species used as experimental animals have the advantages of economy and simple conduct, but they are ill suited for research on human infectious disease. The generation of a new, cost-effective, primate-like, small-animal model would greatly facilitate research into bacterial infectious disease and the development of novel therapeutic approaches to bacterial infection (Niewiesk & Prince, 2002).

Tree shrews are non-rodent, primate-like animals classified into the order Scandentia (Muller et al, 1999). This animal is a native of Yunnan, China, but related species are found all over South-east Asia. There is increasing interest in using them to establish animal models for medical and biological research (Yang et al, 2003) as they are susceptible to infection with a variety of human viruses *in vivo*, including hepatitis (Zhao et al, 2002).

To the best of our knowledge, the establishment of bacterial infection models in tree shrew is rare. In the current study, bacterial counts and histopathological examination were employed to investigate the susceptibility of tree shrew to pathogens, and two bacterial infection models were established using *P. aeruginosa* and *S. aureus* to evaluate the efficacy of antimicrobial agents.

1 Materials and Methods

1.1 Bacterial strains and antibiotics

Strains *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 were obtained from the First Affiliated Hospital of Kunming Medical College (China). Bacteria were cultured in nutrient broth, and incubated at 37 °C overnight. Bacteria were then washed three times with phosphate buffer solution (PBS) by repeated centrifugation for 10 min at 1 000 r/min, and re-suspended in PBS. The CPZ and LEV were obtained from the General Pharmaceutical Factory of Harbin Pharmaceutical Group (Harbin, China). All other reagents were of analytic grade from commercial sources.

1.2 *In vitro* susceptibility of antibiotics

Serial dilution was used as previously described (Zhang et al, 2010). Briefly, minimal inhibitory concentration (MIC) was determined by incubating the bacteria (5×10^5 CFU/mL) in Luria-Bertani (LB) broth (pH 7.0) with variable amounts of the sample tested for 16−18 h at 37 °C. The sample concentrations were the same for each of the tested bacterial strains in three independent experiments and the MIC values were obtained without inter-experiment variations and expressed as μg/mL.

1.3 Animals

Adult tree shrews (*Tupaia belangeri chinensis*) weighing 120−150 g were used in this study. Animals were provided by the Animal Center of the Kunming Institute of Zoology. All animals were housed in individual cages under constant temperature (22 ℃) and humidity with a 12 h light/dark cycle and had *ad libitum* access to chow and water throughout the study. Tree shrews were euthanized by ether inhalation at the completion of the experiments. Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences.

1.4 Burned skin infection model

A third degree burn skin infection model was developed in tree shrews by using *S. aureus* (Dale et al, 2004). Briefly, tree shrews were anesthetized with 90 mg/kg ketamine HCl and 10 mg/kg xylazine. Hair was clipped from the back of anesthetized tree shrews and skin was denuded with 10% Na_2S solution. Then, thermal injury was induced by placing a 90 °C circinal steel (4 cm^2) onto the shaved skin for 10 sec to cause a third degree burn. Immediately after the burn, all tree shrews were given an intraperitoneal injection (i.p.) of 0.5 mL of sterile physiological saline for fluid replacement to prevent overt shock and acetaminophen

(0.25 mg/mL) was given as post burn analgesic in drinking water. Infection was induced by dropping 5×10^6 CFU of *S. aureus* on the surface of the wound. Bacterial counts were conducted 1, 4, and 7 days after infection (n=6).

Twelve hours after inoculation, LEV and CPZ were administrated by subcutaneous injection (s.c.) twice a day for two days at doses of 8 mg/kg and 40 mg/kg body weight, respectively. Bacterial counts were conducted 4 days after infection (n=7).

To characterize the histopathology of the skin infection model in tree shrew (n=4), biopsy specimens were taken 4 days after inoculation. Immediately after the animals were sacrificed, skin samples were taken and immediately fixed in phosphate-buffered (pH 7.4) formalin (10%). The biopsy specimens were embedded in paraffin and stained with hematoxylin and eosin. At least four skin sections per tree shrew were analyzed.

1.5 Graft infection model

Tree shrews were anesthetized as described above, the hair on the back was shaved and the skin cleansed with 10% Na_2S solution. One subcutaneous pocket was made on each side of the median line by a 1.5 cm incision. Aseptically, 1 cm^2 sterile Dacron (BalMedic, china) was implanted into the pockets. Before implantation, the Dacron segments were impregnated with or without 4 mg/mL LEV and CPZ in 30 mg/mL chitosan gel for 30 min, respectively. The pockets were closed by skin clips and a saline solution (0.2 mL) containing 2×10^6 CFU of *P. aeruginosa* 27853 was inoculated onto the Dacron graft surface. The animals were returned to individual cages and acetaminophen (0.25 mg/mL) was given as analgesic in drinking water. At days 1, 3 and 6 after infection, the animals were sacrificed to conduct bacterial counts (n=7). For clinical examination, the wounds of tree shrew were photographed with a digital camera 3 days after infection.

1.6 Bacterial counts

The skin samples were cut and homogenized, and bacterial suspension was prepared for bacterial counts. Dacron grafts were placed in sterile tubes containing 1 mL of phosphate buffer saline and vibrated for 2 min to remove the adherent bacteria. Viable bacteria were obtained by culturing serial 10-fold dilutions (0.1 mL) of the bacterial suspension on LB agar plates at 37 °C for 16 h. The limit of detection for this method was approximately 100 CFU/mL.

1.7 Statistical analysis

The MIC values were calculated as the geometric means of three separate experiments. The significance of differences in mean viable bacterial counts was assessed by the Kruskal-Wallis test. Significance was accepted when the P<0.05.

2 Results

2.1 *In vitro* susceptibility of antibiotics

Both LEV and CPZ are major antimicrobials used in treating bacterial infectious disease. We used the serial dilution method to determine the MIC of antibiotics. Under our experimental conditions, both antibiotics exhibited potent bactericidal activity to *P. aeruginosa* and *S. aureus* (Tab. 1). The MIC data for *P. aeruginosa* and *S. aureus* was in the susceptible range of each antibiotic. However, the MIC of LEV (0.4 μg/mL and 0.8 μg/mL, respectively) to *P. aeruginosa* and *S. aureus* was lower than CPZ (1.6 μg/mL and 3 μg/mL, respectively).

Tab. 1 *in vitro* susceptibility of antibiotics (μg/mL)

	Staphylococcus aureus 25923	*Pseudomonas aeruginosa* 27853
CPZ	3	1. 6
LEV	0. 8	0. 4

2.2 Burned skin infection model

Bacterial counts were used to evaluate the burnt skin infection model. Viable bacteria from the infected skin (100 mg) were $8\log_{10}$ CFU 12 h after application of 5×10^6 CFU of *S. aureus* (Fig. 1). There was no significant difference in the numbers of CFU per wound at 12 h versus 4 days of untreated group. The infection in tree shrew with *S. aureus* persisted 7 days without obvious change. No death occurred in any groups during *in vivo* study.

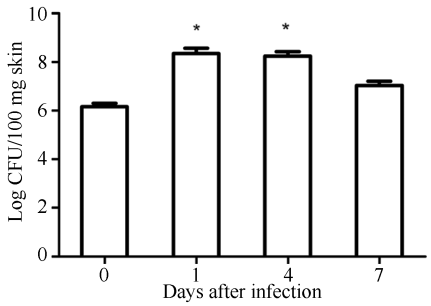

Fig. 1 Bacterial counts in the burnt skin infection model

5×10^6 CFU of *Staphylococcus aureus* were inoculated on the surface of wound after a third degree burn was induced. Bacterial counts were conducted at 0, 1, 4 and 7 d after inoculation. *P<0. 05 versus 3 d post inoculation.

2.3 Dacron graft infection model

No death occurred in any groups in the graft infection model. Bacteria were detected in all tree shrews infected by *P. aeruginosa* at the end of experiment. Inoculation with 2×10^6 CFU/graft of *P. aeruginosa* led to severe infection in the tree shrews (Tab. 2). We observed 7.3$\log_{10}$ CFU/mL of *P. aeruginosa* 3 d after inoculation. Interestingly, there were no obvious infectious symptoms in Kun Ming mice after infection with this dose of *P. aeruginosa* (data not shown). Six days after inoculation, a large numbers of bacteria were observed in the Dacron grafts. In the negative control, however, no bacteria were detected in the Dacron graft without *P. aeruginosa* infection. In addition, obvious infectious symptoms were observed in the photograph of wounds obtained 3 days post inoculation (Fig. 2). Large amounts of pus were observed in the infection group, in contrast, only slight inflammation was observed in the group without infection.

Tab. 2 Bacterial counts in Dacron graft after inoculation of *Pseudomonas aeruginosa*

Group	Mean bacterial counts ($\log_{10}$ CFU/mL)±*SD* (no. of grafts)
Inoculum size	5. 996±0. 5233
1 day post-infection	7. 021±0. 2269[a] (7)
3 days post-infection	7. 329±0. 2878[b] (7)
6 days post-infection	7. 859±0. 2570[c] (6)

[a] P>0. 05 versus inoculum size; [b] P<0. 05 versus inoculum size; [c] P<0. 001 versus inoculum size; [a] P>0. 05 versus 3 days post infection and 6 days post infection.

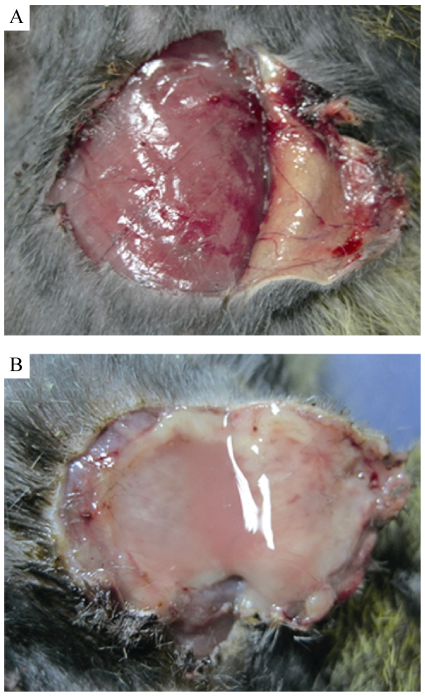

Fig. 2 Clinical examination of Dacron graft infection model induced by *Pseudomonas aeruginosa*

Representative images of non-infected (A) and infected skin (B) 3 days post inoculation.

2.4 Antibiotics are effective in tree shrew infection models

In the burnt skin infection model, LEV (8 mg/kg) and CPZ (40 mg/kg) were injected by s.c. Twelve hours after inoculation. LEV and CPZ reduced the bacterial counts to 4$\log_{10}$ and 5$\log_{10}$ CFU/100 mg skin tissue, respectively. There was no significant difference between LEV and CPZ treatment. However, the differences between the antibiotic treatment group and untreated group were statistically significant (P<0.05) (Fig. 3A).

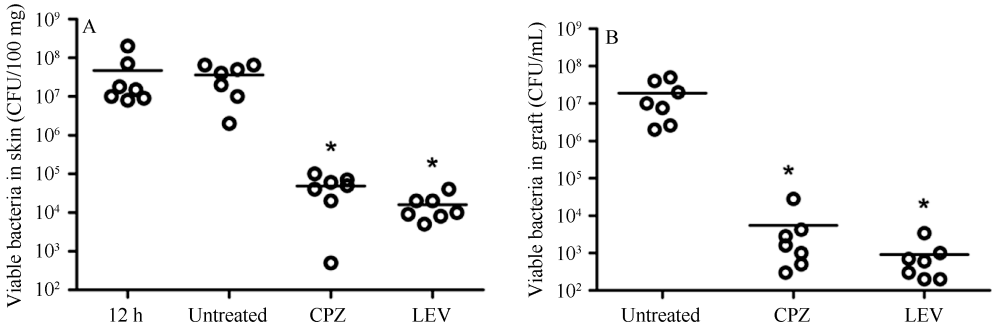

Fig. 3 Efficacy of antibiotics in tree shrew models

(A) Bacterial counts in burnt skin infection model; (B) Bacterial counts in Dacron graft infection model. *P < 0. 05 versus untreated group.

In the Dacron graft infection model, Dacron segments were impregnated with or without 4 mg/mL LEV and CPZ in 30 mg/mL chitosan gel for 30 min. Antimicrobials also prevented infection induced by *P. aeruginosa*. The number of bacteria in the graft were significantly reduced by 4$\log_{10}$ CFU/mL when comparing the antibiotic treatment group with the untreated group (P<0.05) (Fig. 3B).

2.5 Histological examination of burned skin infection model

To characterize the histopathological signs of inflammatory response of the tree shrew after infection

with 5×10^6 CFU of *S. aureus*, skin samples were taken and stained with hematoxylin and eosin. In the burned tree shrew group without infection, the inflammatory response was slight compared with infected tree shrew (Fig. 4B). Obvious histopathological signs of inflammatory response were detected in tree shrews infected with *S. aureus*. Microscopic examination revealed a dense infiltration of leukocytes in subcutaneous tissues and deep dermis. Subcutaneous abscesses were also observed 4 days after infection (Fig. 4C). In contrast, no inflammatory responses were observed in normal skin of tree shrew (Fig. 4A).

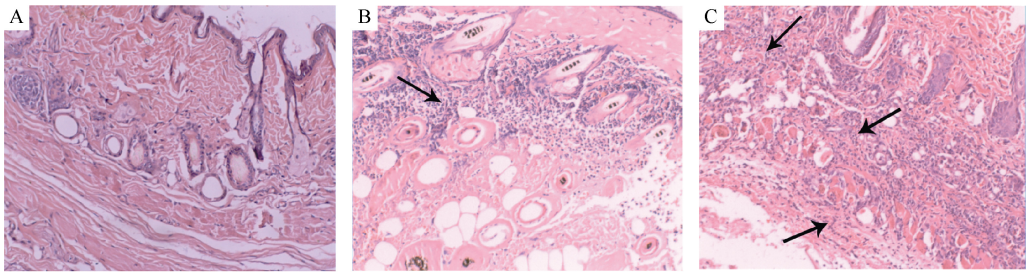

Fig. 4 Histological examination of burnt skin infection model

(A) normal skin; (B) burnt skin; (C) burnt skin with *Pseudomonas aeruginosa* infection. Arrows indicate the infiltration of leukocytes. Selected samples are shown at ×100.

3 Discussion

The efficacy of antimicrobial drugs in animal models is very important during the development of new anti-infectious drugs (Craig, 1993). Some bacterial infection models have been developed in mice, rats, and rabbits, but none have been developed in small primate-like animals (Cao et al, 2003; Wada et al, 2008). Burnt skin infection and graft infection are severe diseases causing persistent infection, even sepsis (Rafla & Tredget, 2011; Turgut et al, 2005). In the present study, we established two bacterial infection models induced by *P. aeruginosa* and *S. aureus* in tree shrew, which is considered as a novel primate-like experimental animal.

Both *P. aeruginosa* and *S. aureus* are major pathogens which cause severe topical and systemic infection in human. These two bacteria are opportunistic, usually causing infections in children and immune compromised patients (Giacometti et al, 2003; Tredget et al, 2004). It is surprising that these bacteria caused severe infection in tree shrew at a low infecting dose. In the burnt skin infection model, $8\log_{10}$ CFU/100 mg skin tissues were detected 12 h after inoculation with 5×10^6 CFU of *S. aureus* per wound and the infection persisted for up to 7 days (Fig. 1). The integrity of skin can block infection induced by bacteria, so we establish this model by inoculating bacteria on the burnt skin. To insure the same burn degree in every tree shrew, the integrity of skin was determined by histological examination. The histopathological signs of inflammatory response were observed by histological examination four days after infection. Additionally, microscopic examination revealed a dense infiltration of leukocytes in subcutaneous tissues and deep dermis (Fig. 4). In the Dacron graft infection model, $7.3\log_{10}$ CFU/mL of *P. aeruginosa* was observed 3 days after the inoculation with 2×10^6 CFU of *P. aeruginosa* per graft (Tab. 2). These two tree shrew infection models caused persistent infection after inoculation with *S. aureus* and *P. aeruginosa*, indicating we successfully established bacterial infection models in tree shrew. Interestingly, the dose of *P. aeruginosa* for the Dacron graft infection model in tree shrew did not induce a persistent infection in Kun Ming mice under our experimental conditions. To induce graft infection in Kun Ming mice with *P. aeruginosa*, more bacteria ($8\log_{10}$ CFU) were needed for Dacron graft infection when compared with tree shrew. The number of infection bacteria is critical for evaluating the therapeutic potential of new antimicrobials. Mice rapidly fight bacteria through their own immune ability, which may be the reason for the difficultly in establishing persistent infection in Kun Ming mice.

Furthermore, both topical treatment and graft pre-coated with antibiotics significantly reduced the bacterial number in the burnt skin infection model and in the Dacron graft infection model (Fig. 3A,B), suggesting the tree shrew bacterial infection models are able to evaluate the efficacy of novel antimicrobials *in vivo*. The results presented in this study show that tree shrew have several advantages as experimental animals for studying bacterial infection and evaluating the efficacy of new antibiotics,

such as: (1) it is susceptible to *P. aeruginosa* and *S. aureus*; (2) it is easy to work with; and (3) antibiotics are effective in bacterial infection tree shrew models.

In conclusion, our findings demonstrate that tree shrew was susceptible to *P. aeruginosa* and *S. aureus* and two bacterial infection models in tree shrew were established, indicating that tree shrew as a new experimental animal is suitable for establishing bacterial infection models and evaluating the efficacy of novel antimicrobial agents.

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