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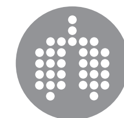

An experimental model of pneumonia induced by methicillin-resistant *Staphylococcus aureus* in ventilated piglets

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ABSTRACT: The objectives of the study were to validate a model of methicillin-resistant *Staphylococcus aureus* (MRSA) pneumonia in ventilated piglets and to study the time-course of biological markers and histopathological changes.

12 piglets were intubated and inoculated with 15 mL of a suspension of 10^6 colony forming units of MRSA in every lobe through the bronchoscope channel. The piglets were ventilated for 12 h (n=6) and 24 h (n=6). Clinical parameters were assessed every 6 h and pro-inflammatory cytokines were measured in serum and in bronchoalveolar lavage (BAL) at baseline and sacrifice. Histopathology of each lobe and cultures from blood, lungs and BAL were performed.

Animals developed histopathological evidence of pneumonia at necropsy. At 12 h, pneumonia was present in all animals and was severe pneumonia at 24 h. Microbiological studies confirmed the presence of MRSA. A significant increase in interleukin (IL)-6, IL-8 and tumour necrosis factor-α values was seen in BAL at 24 h and IL-6 at 12 h. In serum, only IL-6 levels had increased significantly at 24 h.

In ventilated piglets, bronchoscopic inoculation of MRSA induces pneumonia at 12 h and severe pneumonia at 24 h. This severity was associated with a corresponding increase in systemic and local inflammatory response.

KEYWORDS: Animal model, inflammatory response, methicillin-resistant *Staphylococcus aureus*, ventilator-associated pneumonia

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Ventilator-associated pneumonia (VAP) is the primary cause of mortality among nosocomial infections [1]. Many aspects of the diagnosis, pathophysiology and therapy of VAP are not well understood. Animal studies of pneumonia provide a unique opportunity to evaluate some of the incompletely understood mechanisms involved in VAP, without the influence of potential confounders. MARQUETTE *et al.* [2] developed an animal model of pneumonia in mechanically ventilated piglets. This model has proved to be very useful for the proper evaluation of different aspects of VAP [3–9]. Using this animal model, our group assessed the associated local (lung) and systemic (serum) inflammatory response (IR) in experimental VAP caused by *Pseudomona aeruginosa* after 4 days of mechanical ventilation [9]. Although these studies proved the feasibility of performing experiments in animals that are ventilated for a long period of time, they are not exempt from potential shortcomings. Above all, there are the economic expenses derived from a 4-day experiment, with the requirement of specifically trained personnel to properly manage animals that often are in a critical state. Also, the potential of contamination with other microorganisms may reasonably increase over time, further complicating the interpretation of the data. Therefore, shorter experiments would be optimal, particularly if we consider that the precise time-course of both the IR of the host to the microbiological inoculation and the development of histopathological lesions of pneumonia are not known in this animal model. In the present study, we aimed to validate a model of VAP in ventilated piglets, assessing the time-course of relevant biological markers of inflammation and the histopathological changes of the lung 12 and 24 h after the inoculation of methicillin-resistant *Staphylococcus aureus* (MRSA). We chose this microorganism because it is one of the most common causes of nosocomial pneumonia, contributing to significant morbidity and mortality [10–12]. Also, the paucity of antibiotics to treat MRSA infections, their

potential toxicity and variations in lung distribution make animal models particularly attractive for performing pharmacodynamic and pharmacokinetic studies.

METHODS

Experiments were performed in an experimental intensive care unit (ICU) fully equipped with cardiovascular monitors (Hewlett Packard model 658; Hewlett-Packard, Madrid, Spain), ventilators (Servo 900D; Siemens, Madrid, Spain) and electrical infuser pumps (Juac 591, San Diego, CA, USA).

Animal preparation

12 healthy, domestic-bred largewhite–Landrace piglets, 3–4 months of age with a mean±SE body weight of 20±2 kg were used in this study. The animals were sedated by an intramuscular injection (2 mg·kg^{-1}) of azaperone (Stresnil®; Esteve, Barcelona, Spain) and anaesthetised with 30 mg·kg^{-1} intravenous sodium thiopental (Pentotal®; Abbot, Madrid, Spain). They were then orotracheally intubated with a 7.5-mm low-pressure cuff tube (Mallinckrodt 7.5; Mallinckrodt Medical, Athlone, Ireland) and connected to the ventilator. Anaesthesia was maintained with a continuous infusion of midazolam (Midazolam®; Reig-Jofré, Barcelona, Spain) 0.4 mg·kg^{-1}·h^{-1} and phentanyl (Fentanest®, Kern Pharma, Barcelona, Spain) 5 μg·kg^{-1}·h^{-1}, followed by a bolus of intravenous of sodium thiopental. A catheter was inserted in the femoral vein 7 French (F) (Plastimed, Prodimed, St Leu-la-Forêt, France) for continuous infusion of 5% dextrose solution (0.8 mL·kg^{-1}·h^{-1}) and 0.9% saline solution (0.8 mL·kg^{-1}·h^{-1}) with an infusion pump. The femoral artery was cannulated with a 3F polyethylene catheter (Plastimed, Prodimed) for pressure monitoring and blood sampling. An 8F suprapubic urinary catheter (Rüsch, Kammating, Malaysia) was placed in the bladder through surgical midline minipelvitomy. The piglets were then placed in a prone position and were mechanically ventilated for 12 or 24 h.

Mechanical ventilation

Animals were mechanically ventilated in a volume-controlled mode. Ventilator parameters consisted of: tidal volume (V_T) at a constant inspiratory flow of 10 mL·kg^{-1}; a respiratory frequency (f_R) of 15 breaths·min^{-1}; an inspiratory time of 33%; and an initial inspiratory fraction (F_{I,O_2}) of 100% with no positive end-expiratory pressure (PEEP). Later, F_{I,O_2} was set according to blood gas analysis in order to obtain an arterial oxygen tension (P_{a,O_2}) of 80–100 mmHg, and PEEP was increased up to 5 cmH_2O when required. Airway pressures, static lung compliance (calculated by dividing V_T by the difference between the end-inspiratory plateau pressure and the total PEEP [13]) and arterial blood gases (measure using a blood gas analyser, AutomaticQC Cartridge: Siemens, Tarrytown, NY, USA) were determined every 6 h. Throughout the protocol, the arterial carbon dioxide tension was maintained at 35–45 mmHg by increasing the f_R to the maximum level preceding the appearance of auto-PEEP. Above this limit, hypercapnia was tolerated, as described previously [14].

Bronchial inoculation

75 mL of 10^6 colony forming units (cfu)·mL^{-1} of pathogenic MRSA (strain isolated from patients who were Panton Valentine Leukocidin (PVL) status-negative; minimal inhibitory concentration for vancomycin was 1 μg·mL^{-1} and 2 μg·mL^{-1} for linezolid) were inoculated using a fibre bronchoscope and evenly distributed among every lobe of each lung. Inoculations of bacteria were instilled once the animals were haemodynamically stable after sedation and mechanical ventilation.

Sampling and procedures

Mechanical ventilation parameters (V_T, f_R, airway pressures and F_{I,O_2}), cardiac rate, blood pressure, body temperature, blood gases and serum electrolytes (sodium and potassium) were monitored at 0, 6, 12, 18 and 24 h. Blood biochemistry and blood cell counts were obtained at 0, 12 and 24 h.

Bronchoalveolar lavage

Two 15-mL aliquots of sterile saline solution (0.9% sodium chloride) were instilled and re-aspirated through the bronchoscope channel in the right middle lobe at 0 h (before the inoculation of the MRSA suspension) and at sacrifice (12 or 24 h).

Inflammatory parameters

Cytokines in blood and bronchoalveolar lavage fluid

Tumour necrosis factor (TNF)-α and interleukin (IL)-6 and IL-8 levels were measured in serum and bronchoalveolar lavage (BAL) supernatant using the ELISA method in porcine-specific kits (R&D Systems Inc., Minneapolis, MN, USA).

BAL cytokines were determined at the time of intubation and at the end of the study. Serum cytokines were determined at the time of intubation (baseline) and at 12 and 24 h.

Sacrifice and *post mortem* studies

Euthanasia was induced at 12 h in six piglets and at 24 h in the other six piglets. Sacrifice was performed under general anaesthesia by intravenous overdose of potassium chloride (40 mEq, rapid *i.v.*; B. Braun Medical S.A., Barcelona, Spain).

Collection of lung specimens

After death, the animals remained under mechanical ventilation until surgical samples of lung parenchyma were obtained for bacteriological and histopathological evaluation. The lungs were aseptically exposed through a cervicothoracic midline incision. Thereafter, at least one lung tissue specimen (3 cm^3) was taken from both the better preserved lobe (identified macroscopically) and the more involved lobe. Specimens were cut into two parts for bacteriological and pathological studies.

Bacteriological studies

Quantitative cultures of BAL fluid, serum and lung tissue were performed at the end of the study. BAL and lung tissue specimens were processed for quantitative bacterial cultures as in previously published [15], according to recommended laboratory guidelines [16].

Histopathological assessment

Lung tissue was processed in accordance with standard methods. Analyses of vessels (thrombosis and endothelial lesions), pleura (acute or chronic pleuritis) and lung parenchyma were performed. The evaluation of lung parenchyma included severity of pneumonia and the presence of other associated lesions (hyaline membranes and alveolar damage). Severity of pneumonia was graded according to previously published criteria [17] in the following grades. 0=no pneumonia; 1=purulent

mucous plugging; 2=bronchiolitis; 3=pneumonia (consolidation coexisting with significant accumulation of polymorphonuclear leukocytes, fibrinous exudates and cellular debris into the alveolar space); 4=confluent pneumonia (extension along different secondary lobes); and 5=abscessed pneumonia (cellular necrosis coexisting with disruption of cellular architecture). Pneumonia was limited to the last three categories. Classification of each specimen was based upon the worst category observed.

Statistical analysis

All data are expressed as mean±SE. Nonparametric tests for paired data were used; Wilcoxon and Friedman tests were used for comparison of two or more than two time-points, respectively. A p-value of <0.05 was considered statistically significant.

Approval by the institutional committee

The study was approved by the Institutional Review Board and Ethics Committee of Hospital Clinic (Barcelona, Spain); animal care complied with the ''Guide for the Care and Use of Laboratory Animals'' published by the US National Institutes of Health [18], and by local government guidelines.

RESULTS

Physiological and laboratory data

Physiological and laboratory variables are shown in table 1. After bronchial inoculation, a rapid and persistent decrease of the static lung compliance ($p<0.05$) and mean arterial pressure ($p<0.01$), and an increase in body temperature ($p<0.01$) and cardiac rate ($p<0.05$) were observed throughout the study. Moreover, $P_{a,O_2}/F_{I,O_2}$ ratio decreased over time, although differences did not reach statistical significance. No differences in biochemical data were observed.

Inflammatory response

A progressive increase in BAL IL-6 concentrations was observed at 12 h, which increased further at 24 h. BAL IL-8 and TNF-α concentrations also increased significantly compared to baseline, but only at 24 h (fig. 1). Conversely, in serum, only IL-6 levels showed a significant increase at 24 h. The measurement of IL-8 and TNF-α concentrations in serum did not experience any significant change over time (fig. 2).

Microbiological findings

All BAL samples performed at the beginning of the study (before instillation of MRSA) were sterile. By contrast, MRSA was present in all the BAL samples collected at the end of the experiment and also in lung tissue cultures of the 12 piglets evaluated. BAL cultures yielded MRSA in a concentration 10^3 cfu·mL^{-1} in eight piglets and 10^4 cfu·mL^{-1} in four piglets. Lung tissue cultures also showed growth of MRSA in a concentration of 10^3 cfu·g^{-1} in all the samples evaluated (fig. 3). Blood cultures were all negative at 12 h and positive in five out of six piglets at 24 h. *Pasteurella multocida* and coagulase-negative *Staphylococcus* were also detected in several of the samples, although at low counts.

Histopathological findings

Histopathological evaluation of lung samples showed the presence of pneumonia in all the piglets, including those sacrificed at 12 h (grades 3–5). However, severe pneumonia, defined as abscessed or confluent pneumonia (grades 4 and 5), was present in all pigs sacrificed at 24 h and in only one sacrificed at 12 h.

DISCUSSION

The main finding of the present study is that histopathological signs of pneumonia and its associated lung IR are already evident 12 h after the bronchoscopic inoculation of pathogenic MRSA in a ventilated piglet. Both the severity of pulmonary lesions and the intensity of the IR increased further 24 h after inoculation. These findings confirm, for the first time, the potential utility of this model to study the early effects of antibiotic treatments and coadjuvant therapies against severe MRSA pneumonia 12 h after microbial inoculation, decreasing the length and cost of these types of experiments.

Our findings confirm those obtained previously by our own group using *P. aeruginosa* as a microbial aetiological agent of VAP in this animal model [9]. After the inoculation of MRSA, animals developed clinical signs of pneumonia (fever, tachycardia, hypotension and gas exchange impairment) and deterioration in lung mechanics (decrease in static compliance over time). Histopatological signs of pneumonia were already evident at 12 h, although they increased by 24 h, when all the animals evaluated presented severe pneumonia (defined as

TABLE 1 Sequential measurements of physiological and laboratory parameters

	Time h					p-value#
	0	6	12	18	24	
Temperature °C	35.4±0.2	36.7±0.3	37.9±0.3	37.4±0.8	37.9±1.1	**<0.001**
Cardiac frequency beats·min^{-1}	74±4.6	90±8.1	97±5.3	100±8.5	97±8.2	**0.02**
Arterial pressure mmHg	92±3.8	85±4.2	84±4.1	77±7.5	63±5.6	**<0.001**
$C_{L,s}$ mL·cmH$_2$O^{-1}	21.3±2	19.6±1.6	19±1.9	18.8±3.5	15.8±4.1	**0.02**
$P_{a,O_2}/F_{I,O_2}$ mmHg	467.6±22.9	471.4±31	379.1±38.1	335.8±73.9	376.7±92.4	NS
WBC count ×10^9 cells·L^{-1}	11.8±1.3		20.6±2.4		12.6±3.7	0.07
Creatinine mg·dL^{-1}	0.8±0.06		0.8±0.07		0.7±0.05	NS

Data are presented as mean±SE, unless otherwise stated. $C_{L,s}$: static lung compliance; P_{a,O_2}: arterial oxygen tension; F_{I,O_2}: inspiratory oxygen fraction; WBC: white blood cell; NS: not significant. #: calculated using the Friedman test. Bold type indicates $p<0.05$.

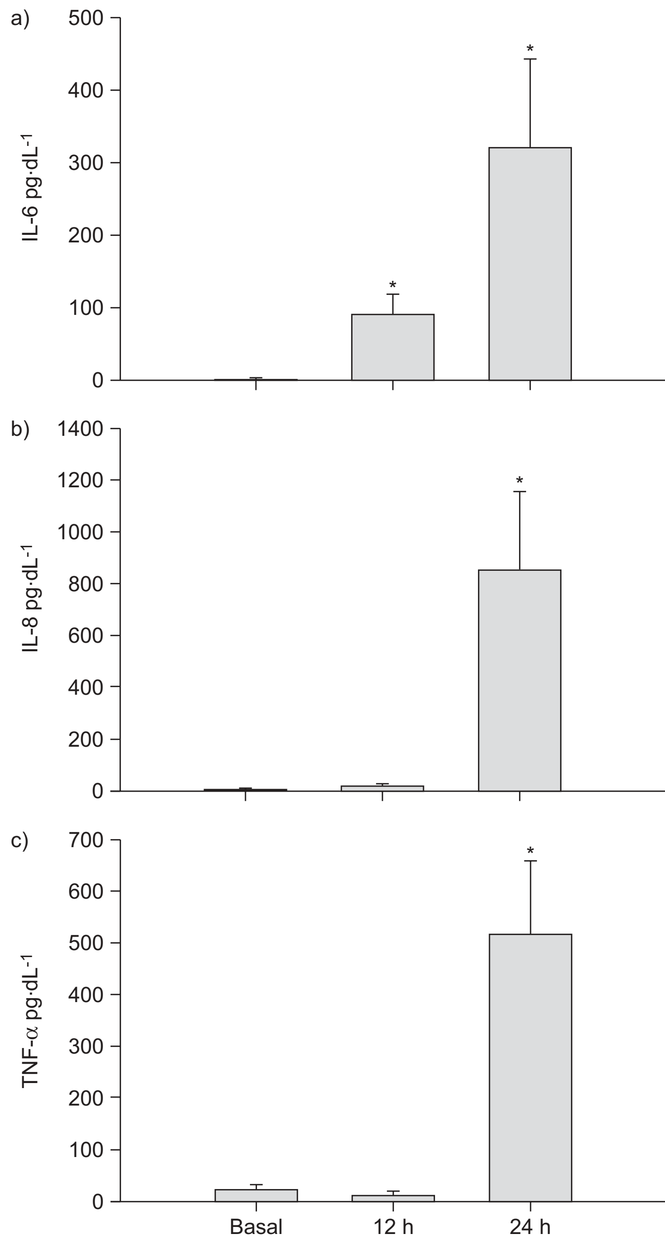

FIGURE 1. Sequential determination of a) interleukin (IL)-6, b) IL-8 and c) tumour necrosis factor (TNF)-α in bronchoalveolar lavage. Data are presented as mean±SE. *: $p<0.05$ (calculated using the Wilcoxon test).

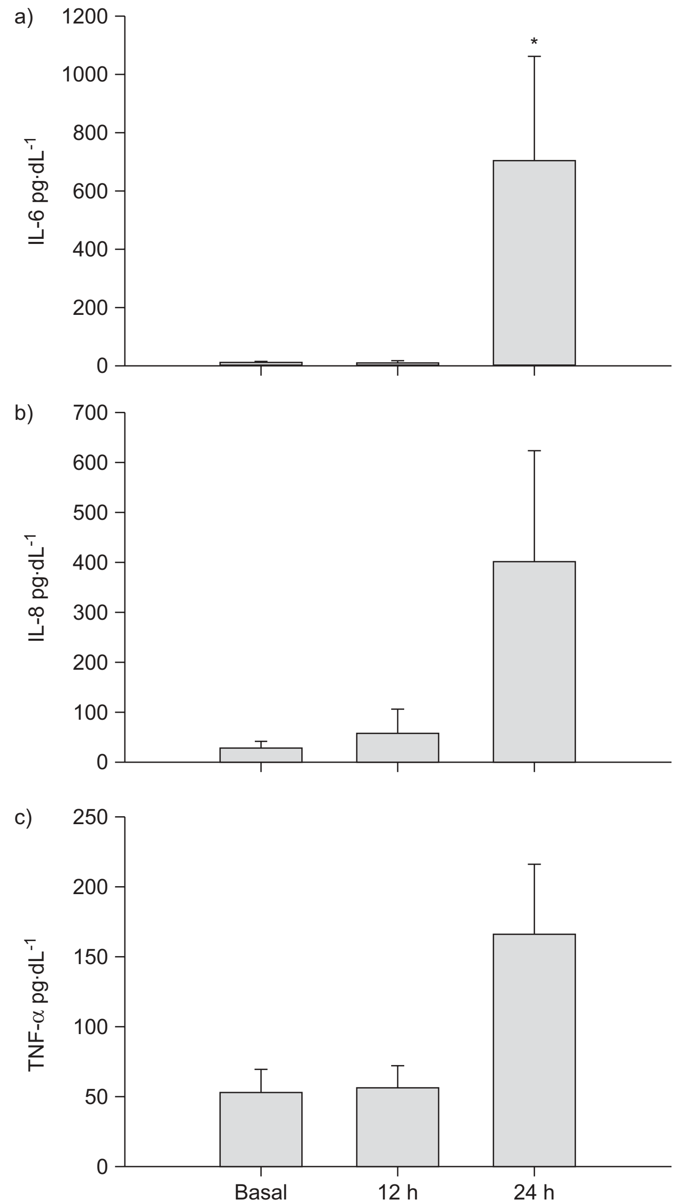

FIGURE 2. Sequential determination of a) interleukin (IL)-6, b) IL-8 and c) tumour necrosis factor (TNF)-α in serum. Data are presented as mean±SE. *: $p<0.05$ (calculated using the Wilcoxon test).

abscessed or confluent pneumonia). Also, this study confirms the compartmentalisation of the IR and the critical role of IL-6 in the associated IR, as previously described in the same animal model [9]. IL-6 was the only cytokine that increased significantly at 12 h in the lung, and the only one that experienced significantly increased levels in serum. Concentrations of IL-8 and TNF-α in BAL increased later on, when the severity of pneumonia progressed. It is noteworthy that IL-6 concentration in serum has previously been shown to be an independent predictive factor of mortality in different population groups [17, 19]. It seems that its determination in serum and BAL might be a very useful parameter to assess the magnitude of the IR and the potential effects of different anti-inflammatory treatments.

In the present study, we chose MRSA as the aetiological agent for several reasons. First, Gram-positive pathogens are being reported with increasing frequency as a cause of nosocomial pneumonia mortality [11]. *S. aureus* is the most common cause of nosocomial pneumonia and the leading cause of death [20]. This microorganism has developed progressive resistance to β-lactam antibiotics since MRSA strains emerged in the 1980s [21, 22]. Since then, many institutions throughout the world have reported outbreaks of nosocomial pneumonia caused by MRSA [23, 24]. It is estimated that the risk of death in MRSA episodes is 20 times higher than episodes caused by methicillin-sensitive *S. aureus* [25, 26]. Due to the increased prevalence of MRSA infection in ventilated patients and the high associated mortality, studies aimed at better knowing

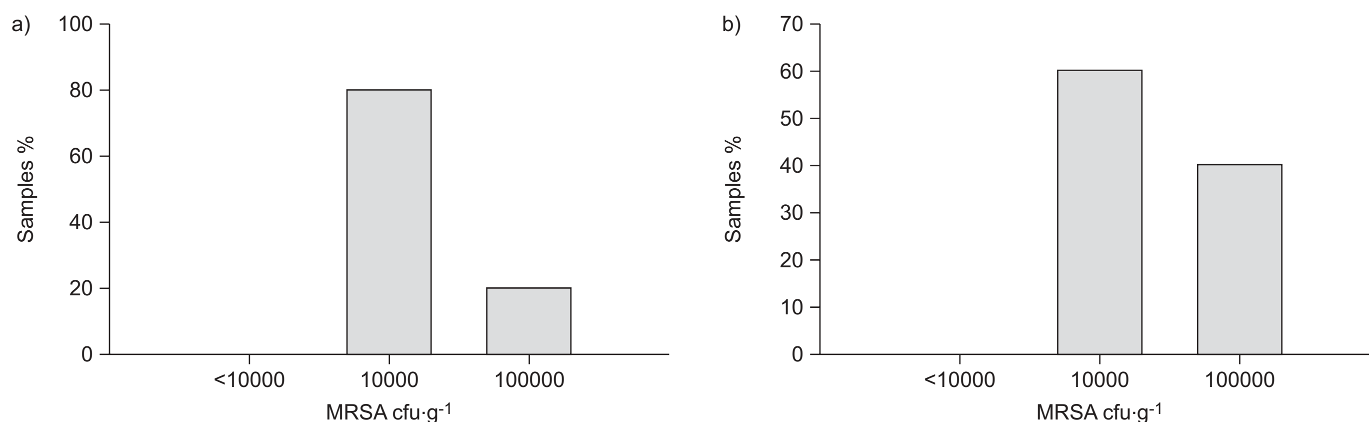

FIGURE 3. Microbiological findings from a) bronchoalveolar lavage and b) lung tissue samples. MRSA: methicillin-resistant *Staphylococcus aureus*; CFU: colony-forming unit.

both the pathogenesis of the infection and the effectiveness of antibiotic treatment is of paramount importance.

Different authors have shown that it is possible to develop VAP when piglets are ventilated for as long as 96 h [2–10, 14]. In the present study, we have shown, for the first time that histopathologically confirmed pneumonia can be obvious as early as 12 h after the inoculation of MRSA. Shortening the experiments has obvious potential advantages. First, savings in both financial and human resources are expected, since the set-up of the experiments requires continuous monitoring of the animals. As a result, a large group of ICU professionals (both physicians and nurses) is required. Secondly, the risk of lung contamination by other microorganisms and the potential development of extrapulmonary infections increase with time, complicating the interpretation of the results obtained. Thirdly, knowing the exact timetable of the appearance of signs of pneumonia after the inoculation of the microorganism may help us to define the precise role of antibiotics in influencing the course of the infection and the associated IR. This point is particularly relevant when considering MRSA pneumonia. The therapeutic options available to treat serious infections due to MRSA are limited [27, 28]. The emergence of MRSA strains with reduced vancomycin susceptibility (SA-RVS) further reduces treatment options. Moreover, the pharmacokinetic profile in ICU patients is frequently too variable to ensure the optimal therapeutic outcome by using the standard antibiotic dosage [29, 30]. Not only pharmocokinetics, but also pharmacodynamics characteristics influence dosing regimens of antimicrobials. Antibiotic concentrations at the site of infection differ greatly from those in plasma, since drug penetration varies depending on the drug, the tissue involved and the infection. Thus, in localised infections, such as pneumonia, it is extremely important to know what fraction of the free drug will be able to cross the membranes and barriers and reach the site of the infection. The availability of an animal model of MRSA pneumonia in which it is possible to perform accurate pharmacokinetic and pharmacodynamic studies, both in serum and in the lung, provides a unique opportunity to gain insight into relevant aspects regarding the response to specific treatments. The increasing recognition of SA-RVS will require controlled studies comparing the efficacy of new therapeutic agents; animal models may also be very useful for this purpose. LUNA *et al.* [31] recently published the potential favourable effects of linezolid compared with glycopeptides in MRSA pneumonia in piglets. Animals treated with linezolid had a better survival and a trend to better clearance of MRSA, not attributable exclusively to pharmacokinetic and pharmacodynamic effects. Further studies of the efficacy of these new agents against infections caused by MRSA or SA-RVS are warranted.

The present study has limitations. First, the exogenous administration of high bacterial inocula in a previously healthy animal does not necessarily reflect the complexities of pneumonia development in humans. Secondly, potential species differences in lung immunology between piglets and humans must also be considered. Finally, this is a relative ''pure'' model of VAP in which animals do not suffer from comorbidities, contrary to what occurs in critically ill patients under mechanical ventilation. However, there are also clear advantages of this animal model, in which the specific role of a particular treatment can be evaluated, and pharmacokinetic and pharmacodynamic evaluations can be performed both in serum and in the lung.

In summary, the present study demonstrates that in ventilated piglets, it is feasible to reproduce MRSA pneumonia and its associated IR 12 h after inoculation, with the potential advantages that shortening the experiments may have.

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STATEMENT OF INTEREST

None declared.

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