AN EXPERIMENTAL MODEL OF PNEUMONIA INDUCED BY METHICILLIN-RESISTENT STAPHYLOCOCCUS AUREUS IN VENTILATED PIGLETS

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INTRODUCTION

Ventilator-associated pneumonia (VAP) is the first cause of mortality among nosocomial infections [1]. Many aspects regarding the diagnosis, pathophysiology, and therapy of VAP remain unsolved. 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]. Our group, using this animal model, assessed the associated local (lung) and systemic (serum) inflammatory response (IR) in experimental VAP induced by *Pseudomonas aeruginosa* after 4 days of mechanical ventilation [10]. Although these studies proved the feasibility of performing animal experiments that are ventilated for a long period of time, they are no exempt of potential shortcomings. Above all, the economic expenses derived from a 4 days long experiment, with the requirement of specific trained personnel to properly manage animals that often are in a critical situation. Also, the potential of contamination with other microorganisms may reasonably increase as the time goes by, further complicating the interpretation of the data. In this sense, 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 hours after the inoculation of *methicillin-resistant Staphylococcus aureus* (MRSA). We chose this microorganism because is one of the most common causes of nosocomial pneumonia contributing to significant morbidity and mortality [11-12]. Also, the paucity of antibiotics to treat MRSA infections, their potential toxicity and variations in lung distribution makes animal models particularly attractive to perform pharmacodinamic and pharmacokinetic studies.
METHODS

Experiments were done in an experimental 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, California, USA).

Animal preparation

Twelve healthy domestic-bred Largewhite-Landrace piglets of 3 to 4 month age and mean body weight of 20±2 kg were used in this study. The animals were sedated by an intramuscular injection (2 mg/kg) of azaperone (Stresnil®, Esteve, Barcelona, Spain) and anesthetized with 30 mg/kg 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 and eventually bolus intravenous of sodium thiopental. A catheter was inserted in the femoral vein 7F (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, St Leu-la-Forêt, France) 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 hours.

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 (fR) of 15 breaths·min⁻¹, an inspiratory time of 33%, with an initial inspiratory fraction (FiO_2) of 100% with no positive end-expiratory pressure (PEEP). Later, FiO₂ was set according to blood gas analysis in order to obtain arterial oxygen tension (PaO₂) >80 and <100 mmHg, and PEEP was increased up to 5 cm H₂O 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 (blood gas analyser, AutomaticQC Cartridge, Siemens, Tarrytown, NY, USA) were determined every 6 h. Throughout the protocol, the carbon dioxide arterial tension was maintained at 35–45 mmHg by increasing the fR to the maximum level preceding the appearance of auto-PEEP. Above this limit, hypercapnia was tolerated, as described previously [14].

**Bronchial inoculation**

Seventy-five mL of $10^6$ colony forming units (cfu)$\cdot$mL$^{-1}$ of pathogenic MRSA (strain isolated from patients with Panton Valentine Leukocidin (PVL) status negative and minimal inhibitory concentration (MIC) for vancomycin was 1 mcg$\cdot$mL$^{-1}$ and 2 mcg$\cdot$mL$^{-1}$ for linezolid), were inoculated using a fiberbronchoscope and evenly distributed among every lobe of each lung. Inoculations of bacteria were instilled once the animals were haemodinamically stable after sedation and mechanical ventilation.

**Sampling and procedures**

Mechanical ventilation parameters ($V_T$, fR, airway pressures and FiO$_2$), cardiac rate, blood pressure, body temperature, blood gases, serum electrolytes (sodium, potassium) were monitored at 0, 6, 12, 18 and 24 h. Blood biochemistry and blood cells count 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’s 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 BAL fluid:*

Tumour necrosis factor (TNF-$\alpha$) and interleukin IL-6 and IL-8 levels were measured in serum and BAL supernatant using the ELISA method in specific porcine 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 6 piglets and at 24 h in the other 6 piglets. Sacrifice was done under general anaesthesia by intravenous overdose of potassium chloride (40 mEq, rapid iv; B. Braun Medical S.A.).

**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 more preserved lobe (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 previously published [15], according to recommended laboratory guidelines [16].

**Histopathological assessment**
Lung tissue was processed in according with standard methods. Analysis of vessels (thrombosis and endothelial lesions) and pleural (acute or chronic pleuritis) and lung parenchyma was performed. The evaluation of lung parenchyma included severity of pneumonia and 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 were expressed as mean± standard error (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 < 0.05 was considered statistically significant.

**Approval by the Institutional Committee**
The study was approved by the Institutional Review Board and Ethics Committee of our Institution; animal care complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), 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), mean arterial pressure (p<0.01), a rise in body temperature (p<0.01) and cardiac rate (p<0.05) were observed throughout the study. Moreover, PaO$_2$/FiO$_2$ ratio decreased over time, although differences did not reach statistical significance. No differences in biochemical data were observed.

Inflammatory response
Regarding the local IR, a progressive raise in BAL IL-6 concentrations were observed at 12h, which increased further at 24h. Concerning BAL IL-8 and TNF-α concentrations, they also increased significantly compared to baseline but only at 24h (fig. 1). By contrast, in serum, only IL-6 levels showed a significant increase at 24h. The measurement of IL-8 and TNF-α concentrations in serum did not experience any significant change over time (fig. 2).

Microbiology 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 twelve 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 $10^3$cfu·g$^{-1}$ in all the samples evaluated (fig. 3). Blood cultures were all negative at hour 12 and positive in 5 out of 6 piglets at hour 24. Pasteurella multocida and Staphylococcus coagulase-negative were also detected in several of the samples, although in low counts.

Histopathological findings
Histopathological evaluation of lung samples evidenced the presence of pneumonia in all the piglets, including those sacrificed at 12h (grades 3–5). However, severe pneumonia, defined as abscessed or confluent pneumonia
(grades 4 and 5), was present in all pigs sacrificed at 24 hours and in only one sacrificed at 12 hours.
DISCUSSION
The main finding of the present study is that histopathological signs of pneumonia and its associated lung inflammatory response (IR) are already evident 12h after the bronchoscopic inoculation of pathogenic MRSA in a ventilated piglet. Both, the severity of pulmonary lesions and the intensity of the IR further increase 24h after the 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 after 12h of microbial inoculation, decreasing in that way the length and cost of these types of experiments.

Our findings confirm those obtained previously by our own group using P. aeruginosas as microbial aetiology of VAP in this animal model (10). Thus, 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). Histopathological signs of pneumonia were already evident at 12h although increased at 24h, when all the animals evaluated presented severe pneumonia (defined as abscessed or confluent pneumonia). Also, this study confirms the compartmentalization of the IR and the critical role of IL-6 in the associated IR, as previously described in the same animal model [10]. IL-6 was the only cytokine that increased significantly at 12h in the lung, and the only one that experience significant increased levels in serum. Concentrations of IL-8 and TNF-α in BAL, increased later on, when the severity of pneumonia progressed. To note that IL-6 concentration in serum has been shown to be an independent predictive factor of mortality in different population groups [17-18]. 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 have chosen MRSA as etiological agent for different reasons. First, Gram-positive pathogens are being reported with increasing frequency as a cause of nosocomial pneumonia mortality [11]. Particularly, Staphylococcus aureus is the commonest cause of nosocomial pneumonia and the leading cause of death [19]. This microorganism has developed progressive resistance to β-lactams, and MRSA strains emerged in the 1980s [20-21]. Since
then, many institutions throughout the world have reported outbreaks of nosocomial pneumonia caused by \textit{MRSA} [22-23], it is estimated that the risk of death in \textit{MRSA} episodes is 20 times higher than in episodes caused by methicillin-sensitive \textit{S. aureus} [24-25]. Due to the increased prevalence of \textit{MRSA} infection in ventilated patients and the high associated mortality, studies aimed at better knowing both, the pathogenesis of the infection and the effectiveness of antibiotic treatment are of paramount importance.

Different authors have shown that it is possible to develop VAP when piglets are ventilated for as long as 96h [2-10,14]. In the present study we have evidenced, for the first time, that histopathological confirmed pneumonia can be obvious as early as 12h after the inoculation of \textit{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) are required. Second, the risk of lung contamination by other micro-organisms and the potential development of extrapulmonary infections increase with time, complicating the proper interpretation of the results obtained. Third, knowing the exact timetable of the apparition of signs of pneumonia after the inoculation of the microorganism may help us to define the precise role of antibiotics in influencing the curse of the infection and the associated IR. This point is particularly relevant when considering \textit{MRSA} pneumonia. The therapeutic options available to treat serious infections due to \textit{MRSA} are limited [26-27]. The emergence of \textit{MRSA} strains with reduced vancomycin susceptibility (\textit{SA-RVS}) further reduces treatment options. Moreover, the pharmacokinetic (PK) profile in ICU patients is frequently too variable to assure the optimal therapeutic outcome by using the standard antibiotic dosage [28-29]. Not only PK but also pharmacodynamics (PD) characteristics influence dosing regimens of antimicrobials. Antibiotic concentrations at the site of the infection differ greatly from those in plasma since drug penetration varies depending on the drug, the tissue involved and also the infection. Thus, in localized infections such as pneumonia, it is extremely important to know what fraction of the free drug will be able to cross the membranes/barriers and reach the site of the infection. The availability of an animal model of \textit{MRSA} pneumonia in which it is possible to perform accurate
PK and PD 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, and animal models can be very useful for this specific purpose. In this sense, Luna et al [30] 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 PK/PD 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 highly bacterial inoculums in a previously healthy animal does not necessarily reflect the complexities of pneumonia development in humans. Second, 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 PK/PD evaluations can be performed both in serum and in the lung.

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


**TABLE 1:** Sequential measurements of physiological and laboratory parameters during the study.

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>6 h</th>
<th>12 h</th>
<th>18 h</th>
<th>24 h</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Temperature ºC</td>
<td>35.4±0.2</td>
<td>36.7±0.3</td>
<td>37.9±0.3</td>
<td>37.4±0.8</td>
<td>37.9±1.1</td>
<td>&lt;0.001</td>
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<tr>
<td>Cardiac rate bpm</td>
<td>74±4.6</td>
<td>90±8.1</td>
<td>97±5.3</td>
<td>100±8.5</td>
<td>97±8.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Arterial pressure mmHg</td>
<td>92±3.8</td>
<td>85±4.2</td>
<td>84±4.1</td>
<td>77±7.5</td>
<td>63±5.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CL,s mL·cmH₂O⁻¹</td>
<td>21.3±2</td>
<td>19.6±1.6</td>
<td>19±1.9</td>
<td>18.8±3.5</td>
<td>15.8±4.1</td>
<td>0.02</td>
</tr>
<tr>
<td>PaₐO₂/FI,O₂ mmHg</td>
<td>467.6±22.9</td>
<td>471.4±31</td>
<td>379.1±38.1</td>
<td>335.8±73.9</td>
<td>376.7±92.4</td>
<td>ns</td>
</tr>
<tr>
<td>WBC count x10⁹ cells·L⁻¹</td>
<td>11.8±1.3</td>
<td>20.6±2.4</td>
<td>12.6±3.7</td>
<td>ns</td>
<td></td>
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</tr>
<tr>
<td>Creatinine mg·dL⁻¹</td>
<td>0.8±0.06</td>
<td>0.8±0.07</td>
<td>0.7±0.05</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean±SE, unless otherwise stated. bpm: beats per minute; PaO₂: arterial oxygen tension; CL,s: static lung compliance; FI,O₂: inspiratory fraction; WBC: white blood cell; #: calculated according to the Friedman test. 1 mmHg = 0.133 kPa.
Fig. 1. Sequential determination of BAL a) IL-6, b) IL-8 and c) TNF-α during the study. Data are presented as mean±SE. *Statistical significantly (calculated according to Wilcoxon test).
Fig. 2. Sequential determination of serum a) IL-6, b) IL-8 and c) TNF-α during the study. Data are presented as mean±SE. *statistical significantly (calculated according to Wilcoxon test).

**IL-6 serum**

**IL-8 serum**

**TNF-alfa serum**

Microbiological findings:

**BAL**

**Lung tissue**