A Novel Porcine Model of Ventilator-associated Pneumonia Caused by Oropharyngeal Challenge with *Pseudomonas aeruginosa*

Gianluigi Li Bassi, M.D., Ph.D., Montserrat Rigol, D.V.M., Ph.D., Joan-Daniel Marti, R.P.T., Ph.D.,
Lina Saucedo, M.D., Otavio T. Ranzani, M.D., Ignasi Roca, Ph.D., Maria Cabanas, M.D.,
Laura Muñoz, B.Sc., Valeria Giunta, M.D., Nestor Luque, M.D., Mariano Rinaudo, M.D.,
Mariano Esperatti, M.D., Laia Fernandez-Barat, Ph.D., Miquel Ferrer, M.D., Ph.D., Jordi Vila, Ph.D.,
Jose Ramirez, M.D., Ph.D., Antoni Torres, M.D., Ph.D.

ABSTRACT

Background: Animal models of ventilator-associated pneumonia (VAP) in primates, sheep, and pigs differ in the underlying pulmonary injury, etiology, bacterial inoculation methods, and time to onset. The most common ovine and porcine models do not reproduce the primary pathogenic mechanism of the disease, through the aspiration of oropharyngeal pathogens, or the most prevalent human etiology. Herein the authors characterize a novel porcine model of VAP due to aspiration of oropharyngeal secretions colonized by *Pseudomonas aeruginosa*.

Methods: Ten healthy pigs were intubated, positioned in anti-Trendelenburg, and mechanically ventilated for 72 h. Three animals did not receive bacterial challenge, whereas in seven animals, a *P. aeruginosa* suspension was instilled into the oropharynx. Tracheal aspirates were cultured and respiratory mechanics were recorded. On autopsy, lobar samples were obtained to corroborate VAP through microbiological and histological studies.

Results: In animals not challenged, diverse bacterial colonization of the airways was found and monolobar VAP rarely developed. In animals with *P. aeruginosa* challenge, colonization of tracheal secretion increased up to 6.39 ± 0.34 log colony-forming unit (cfu)/ml ($P < 0.001$). VAP was confirmed in six of seven pigs, in 78% of the cases developed in the dependent lung segments (right medium and lower lobes, $P = 0.032$). The static respiratory system elastance worsened to 41.5 ± 5.8 cm H_2O/l ($P = 0.001$).

Conclusions: The authors devised a VAP model caused by aspiration of oropharyngeal *P. aeruginosa*, a frequent causative pathogen of human VAP. The model also overcomes the practical and legislative limitations associated with the use of primates. The authors' model could be employed to study pathophysiologic mechanisms, as well as novel diagnostic/preventive strategies. (ANESTHESIOLOGY 2014; 120:1205-15)

VENTILATOR-ASSOCIATED pneumonia (VAP) is a frequent respiratory complication in the intensive care unit,[1] associated with increased morbidity and healthcare costs.[2–4] VAP is commonly caused by aerobic Gram-negative bacteria, and among these pathogens *Pseudomonas aeruginosa* is the most prevalent.[3]

Animal models of VAP can be employed to study the pathophysiology of the disease and to evaluate the efficacy and safety of preventive and therapeutic interventions. Throughout the years, several animal models have been developed, which differ considerably in the underlying pulmonary injury, causative pathogens, methods for bacterial inoculation, and time to onset of the disease.[5]

What We Already Know about This Topic

- Former porcine and ovine models of ventilator-associated pneumonia did not closely reproduce etiology and pathogenic mechanisms of human ventilator-associated pneumonia

What This Article Tells Us That Is New

- A novel model of ventilator-associated pneumonia, caused by oropharyngeal instillation of *Pseudomonas aeruginosa* was developed in tracheally intubated pigs, positioned in anti-Tredelenburg

In the '80s, Johanson *et al.*[6–8] described a fundamental model of VAP in intubated baboons (*Papio papio*) with acute

---

Corresponding article on page 1075. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). The first two authors equally contributed to this work (G.L.B. and M.R.).

lung injury, and on mechanical ventilation (MV) up to 8 days. VAP was mostly caused by *P. aeruginosa* and *Staphylococcus aureus*.[6] Nevertheless, new VAP models in pigs and sheep gradually replaced the use of primates in this field of investigation. In particular, Marquette *et al.*[9,10] developed a model of tracheobronchial stenosis and VAP in pigs on MV for 4 days. In a later report by the same group, mechanically ventilated pigs without tracheobronchial injury developed VAP in 90% of cases.[11] Importantly, in these porcine models, *Pasteurella multocida* and *Streptococcus suis* were the predominant causative organisms. Similar colonization by endogenous oropharyngeal pathogens was demonstrated in models developed at the National Institutes of Health in healthy sheep[12,13] and pigs.[14] In these latter models, the animals were positioned with a tracheal orientation above horizontal to enhance microaspiration of oropharyngeal pathogens. Finally, a few investigators focused on the human etiology of VAP and induced severe pneumonia in healthy pigs through massive bronchial inoculation of *Escherichia coli*,[15,16] *P. aeruginosa*,[17] and methicillin-resistant *S. aureus*.[18]

Importantly, the aforementioned ovine and porcine models do not reproduce the most prevalent human etiology,[9,11–14] or the primary pathogenic mechanism of the disease,[15–18] *via* aspiration of colonized oropharyngeal contents. Therefore, the primary aim of this study was to characterize a novel animal model of VAP, which reflected the key pathogenic mechanisms of the disease, in pigs challenged with *P. aeruginosa* into the oropharynx. Additionally, we assessed in a limited number of animals, the endogenous colonization of the respiratory tract and development of VAP during antibiotic treatment and in the absence of bacterial challenge.

Materials and Methods

The Institutional Review Board and Animal Ethics Committee of the University of Barcelona, Barcelona, Spain, approved the protocol. Animals were managed according to the National Institutes of Health guidelines for the use and care of animals.[19]

Animals with Oropharyngeal P. aeruginosa Challenge

Seven female Large White–Landrace pigs (weight, 30.5 ± 1.8 kg; range, 27 to 33 kg) were premedicated with an intramuscular dose of 2 mg/kg of azaperone and induced with an intravenous loading dose of 8 mg/kg sodium thiopental. Pigs were then orotracheally intubated with a 7.5-mm I.D. endotracheal tube (ETT) comprising a high-volume low-pressure cuff (Hi-Lo; Covidien, Boulder, CO). The ratio between the ETT cuff outer (OD) diameter and tracheal internal diameter was 1.9 ± 0.8 (see Supplemental Digital Content 1, http://links.lww.com/ALN/B41, which describes methods to compute this ratio). Animals were connected to a SERVO-i mechanical ventilator (Maquet, Wayne, NJ). A continuous infusion of midazolam (1 to 2.5 mg kg⁻¹ h⁻¹) and fentanyl (5 to 10 µg kg⁻¹ h⁻¹) was administered. Boluses of 50 mg of intravenous sodium thiopental were administered as needed to optimize sedation. A loading dose of vecuronium (0.4 mg/kg), followed by a maintenance dose of 0.3 to 0.4 mg kg⁻¹ h⁻¹ was administered to produce muscle paralysis. We surgically cannulated the femoral artery as previously reported.[20] A 7-French Swan–Ganz catheter (Edwards Lifesciences, Irvine, CA) was inserted into the jugular vein. A no. 8 Foley catheter was introduced into the bladder through surgical minipelvectomy. After surgical preparation, the pigs were placed in the prone position and the custom-made surgical bed was oriented approximately 30 degrees in the anti-Trendelenburg position, to model the semirecumbent position in humans (fig. 1). A fluoroscopic image was taken to assess tracheal orientation. Fluid balance was maintained through infusion of lactated Ringer's and 0.9% NaCl solutions. In order to prevent pneumonia caused by endogenous oropharyngeal flora, 1 g of ceftriaxone was administered intravenously 30 min before intubation, and then 50 mg/kg every 12 h for the entire duration of the study. Every 24 h, arterial and mixed venous blood gases, pulmonary mechanics, hemodynamics, urine output, and ventilatory settings were assessed. Additionally, complete blood count was obtained and reviewed.

Bacterial Challenge. Before bacterial instillation and every 24 h thereafter, we took arterial blood and tracheal secretions for *P. aeruginosa* cultures. Each animal was challenged immediately after surgical preparation and 4 h thereafter. Five milliliter of approximately 10^7 to 10^8 colony-forming unit (cfu)/ml of a log-phase culture of *P. aeruginosa* was slowly instilled, over 5 min, into the oropharynx of the animals. We employed a respiratory isolate of ceftriaxone-resistant *P. aeruginosa*, derived from *P. aeruginosa* ATCC 27853 and selected in a multistep process, under increasing concentrations of ceftriaxone, until reaching a maximum of 256 mg/l. Before each challenge, and up to 10 min thereafter, 5 cm H_2O of positive end-expiratory pressure (PEEP) was applied and the internal cuff pressure was increased to 40 cm H_2O to avoid rapid aspiration of pathogens.

Mechanical Ventilation and Airway Management. Pigs were mechanically ventilated in volume control, tidal volume (V_T) of 10 ml/kg, FIO_2 0.4, no PEEP, and respiratory rate adjusted to maintain $PaCO_2$ between 40 and 45 mmHg. Inspiratory gases were conditioned through a Conchatherm III heated humidifier (Hudson RCI, Temecula, CA). Internal ETT cuff pressure was maintained at 28 cm H_2O through an external mechanical device.[21] Open endotracheal suctioning *via* a 12-French sterile suction catheter (Oppo-Cath Suction Catheter; Pennine Healthcare Ltd., Derby, United Kingdom) was performed every 6 h, or when considered clinically indicated. During the procedure, the catheter was gently inserted into the ETT and slowly advanced up to the proximal trachea. Then the catheter was withdrawn applying intermittent suction and continuous twisting motion. On each tracheal suctioning, a score from 0 to 4 (none, scant, minimal, moderate, large) was used to estimate the quantity of aspirated secretions. Additionally, quality of airway secretions (normal or purulent) was recorded.

Fig. 1. Animal position during the 72-h study. Pigs were placed in the prone position, and the custom-made surgical bed was oriented approximately 30 degrees in the anti-Trendelenburg position to achieve an orientation of the respiratory system as in the semirecumbent position in humans. Additionally, to prevent the downward slide of the animal, the bed was covered by an anti-slip surface and the rear legs were further secured to the bed through cohesive bandage. A fluoroscopic image was taken to evaluate tracheal orientation in this position.

Respiratory Measurements. Airway pressure and respiratory flow rates were measured as previously reported.[20] Flow and pressure signals were recorded on a personal computer for subsequent analysis with dedicated software (Colligo; Elekton, Milan, Italy). The static elastance of the respiratory system, total inspiratory resistance, inspiratory airflow resistance, and inspiratory tissue resistance were calculated through the rapid occlusion method by using standard formulae.[20]

Hemodynamic Measurements. Arterial and venous pressures were measured with disposable pressure transducers (True-Wave Pressure Transducer; Edwards Lifescience). Through the Swan–Ganz catheter pulmonary artery pressure, central venous pressure, pulmonary artery wedged pressure, core blood temperature, and cardiac output were measured. The systemic and pulmonary vascular resistances and venous admixture were calculated using standard formulae.[9]

Autopsy, Microbiological and Histological Studies, and VAP Definition. Seventy-two hours after tracheal intubation (68 h after bacterial challenge), the animal was euthanized. The ETT was clamped and the internal cuff pressure increased to 40 cm H_2O. The animal was positioned supine and, under strict asepsis, the lungs were exposed, excised, and placed on sterile drapes. We took two samples from the most affected region of each of the five lobes (fig. 2) for histological and microbiological assessments. Lung histology was evaluated according to previously published methods and a six-point injury score.[22] Quantitative cultures of Gram-negative pathogens in MacConkey agar were performed using standard methods.[13] Ultimately, bacteria were identified by mass spectrometry through a Microflex LT (Bruker Daltonics GmbH, Leipzig, Germany) and bacterial identification was performed using the MALDI BioTyper 2.0 software (BrukerDaltonics). We assessed the clinical pulmonary infection score at baseline and before autopsy as previously reported[23] (see Supplemental Digital Content 2, http://links.lww.com/ALN/B42, which depicts in table 1 the variables to calculate the modified clinical pulmonary infection score). VAP was confirmed according to a histological injury score of 3 or greater, associated with a quantitative *P. aeruginosa* lobar culture of 3 log cfu/g or greater.[24]

Animals without Oropharyngeal* P. aeruginosa *Challenge

We assessed in three female Large White–Landrace pigs (weight, 31.6 ± 1.5 kg; range, 30 to 33 kg) the endogenous
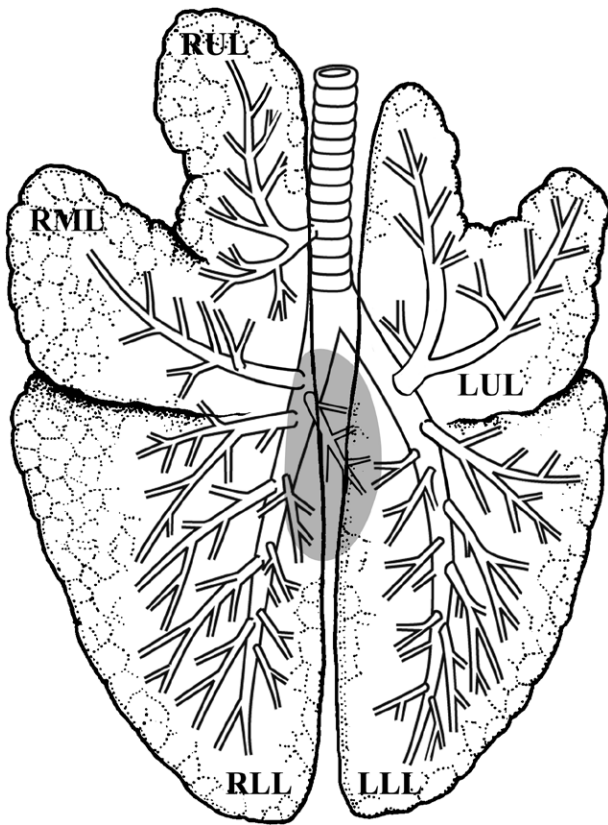
Fig. 2. Anatomy of the pig lungs. The right lung consists of the upper, middle, caudal, and accessory lobes, whereas the left lung consists of the upper and caudal lobes. The left lung is ventilated through the left bronchus. The right main bronchus ventilates the middle, lower, and accessory lobe; whereas the tracheal bronchus, which arises from the right side of the trachea ventilate the upper right lobe. LLL = left lower lobe; LUL = left upper lobe; RLL = right lower lobe; RML = right medium lobe; RUL = right upper lobe.

colonization of the respiratory tract and development of VAP during antibiotic treatment and in the absence of *P. aeruginosa* oropharyngeal challenge. The animals were prepared and managed as described in the section Animals with oropharyngeal *P. aeruginosa* challenge. Upon autopsy, lung samples were plated in MacConkey and blood agar using standard microbiological methods.[13] Bacteria were identified as described in section Animals with oropharyngeal *P. aeruginosa* challenge.

Statistical Analysis. Data are reported as the mean ± SD, unless otherwise specified. One-way repeated measures ANOVA and Friedman test were employed to analyze effects of time on normally or not-normally distributed continuous variables. *Post hoc* multiple comparisons were analyzed using paired Student t tests and Wilcoxon signed ranks test, with Bonferroni correction. To assess the differences in bacterial concentrations and histologic scores between lobes, a restricted maximum likelihood analysis was used, which contained both fixed (lobar colonization) and random effects (subject). If the overall F test was significant ($P \leq 0.05$), multiple comparisons between lobes were tested,

using Bonferroni correction to control for the experiment-wise error rate. Categorical variables were analyzed using Fisher exact test. A two-sided P value less than 0.05 was considered statistically significant. All statistical analyses were performed using SAS software (version 9.2; SAS Institute, Cary, NC).

Results

Animals with Oropharyngeal P. aeruginosa *Challenge*

Six of the seven studied pigs completed the 72-h study; one pig was euthanized after 57 h due to severe respiratory and hemodynamic instability. Pigs were placed on a surgical bed oriented 27.3 ± 1.4 degrees above horizontal, resulting in a tracheal orientation of 13.4 ± 1.5 degrees.

Clinical Findings, Lung Function, and Hemodynamics. Data are reported in table 1. Pigs were generally normothermic, although slightly hypothermic after surgery—the highest mean body temperature was 38.6° ± 0.9°C, after 48 h of tracheal intubation. Leukocyte count did not significantly increase. We aspirated increased amount of tracheal secretions over time, which appeared progressively infected; thus, after 72 h, 80% of the aspirated secretions were purulent. Throughout the study period, the hemodynamic parameters remained stable. The mean respiratory rate was 17.1 ± 1.2 breaths/min (range, 14 to 20 breaths/min) and V_T 304.8 ± 18.5 ml (range, 270 to 330 ml). Pao_2/Fio_2 decreased up to 309.2 ± 107.1, after 48 h of tracheal intubation ($P = 0.004$). The static elastance of the respiratory system worsened during the study reaching 41.5 ± 5.8 cm H_2O/l after 72 h. The clinical pulmonary infection score at baseline and before the autopsy was 0.85 ± 0.37 and 7.0 ± 1.82, respectively ($P = 0.016$) (table 2).

Microbiological and Histological Findings. At the beginning of the study, tracheal secretions were not colonized by *P. aeruginosa* (fig. 3). Conversely, after bacterial challenge the concentration of *P. aeruginosa* in the tracheal secretions progressively increased, eventually reaching 6.39 ± 0.34 log cfu/ml at the end of the study. *Bordetella bronchiseptica* and *E. coli* were seldom found in tracheal secretions (table 2). After bacterial challenge, blood cultures were positive in 4 of 40 samples.

In lobar cultures, *P. aeruginosa* was the only isolated pathogen. The right medium lobe (RML) presented the highest *P. aeruginosa* burden (4.14 ± 1.01 log cfu/g; fig. 4A). Likewise, histological assessment showed, on average, a pattern of confluent pneumonia in the aforementioned lobe (histologic injury score: 4.14 ± 0.69; figs. 4B and 5). Accordingly, VAP was confirmed in six of seven pigs. In one pig, abscessed histological pneumonia was found in the RML, nevertheless VAP was not ultimately confirmed due to a *P. aeruginosa* tissue concentration of 2.86 log cfu/g. VAP developed in the RML in 55.6% of the cases; in the right lower lobe in 22.2%; in the left upper or lower lobes in 11.1%; and never in the right upper lobe ($P = 0.032$). Multilobar infection was found in three of the six pigs that developed

Table 1. Sequential Assessments of Laboratory and Physiological Parameters

	Time (Hours)				
	0	24	48	72	*P* Value
Clinical signs					
Body temperature (°C)	34.7±0.5 (5)	38.2±0.3 (7)	38.6±0.9 (7)	37.7±0.9 (6)	0.011
Leukocytes (10^3/µl)	16.4±5.6 (7)	18.3±9.9 (7)	13.8±5.6 (7)	17.2±7.5 (5)	0.245
Estimated quantity of tracheal secretions	1.3±0.5 (14)	1.8±0.9 (30)	2.5±1.1 (31)	2.4±0.9 (5)	<0.001
Incidence of purulent secretions	0 (14)	17.2 (29)	77.4 (31)	80 (5)	<0.001
Pulmonary gas exchange and mechanics					
Pa_{O_2}/F_{IO_2}	495.7±54.1 (7)	474.8±73.5 (7)	309.2±107.1 (7)	355.9±82.9 (5)	0.004
Pulmonary shunt (%)	3.2±2.1 (6)	2.4±1.3 (6)	9.2±10.1 (7)	5.6±2.4 (4)	0.066
Paw plateau (cm H_2O)	11.2±0.9 (7)	10.9±1.7 (7)	12.3±1.8 (7)	12.5±1.5 (6)	0.067
Elastance respiratory system (cm H_2O/l)	33.2±3.7 (7)	35.6±5.4 (7)	40.2±7.1 (7)	41.5±5.8 (6)	0.001
R_{TOTrs} (cm H_2O l^{-1} s^{-1})	11.6±2.9 (7)	12.5±1.8 (7)	14.7±5.3 (7)	13.9±4.2 (7)	0.736
R_{intrs} (cm H_2O l^{-1} s^{-1})	7.0±2.2 (7)	7.8±0.9 (7)	7.4±1.3 (7)	7.0±0.5 (6)	0.079
ΔR_{rs} (cm H_2O l^{-1} s^{-1})	4.6±1.7 (7)	4.7±2.2 (7)	7.3±5.1 (7)	6.8±4.3 (6)	0.491
Hemodynamics					
HR (beats/min)	54.4±6.4 (7)	71.7±16.7 (7)	59.6±12.8 (7)	57.2±21.9 (6)	0.145
MAP (mmHg)	70.1±10.4 (7)	75.8±8.3 (7)	71.6±10.1 (7)	70.3±6.5 (6)	0.475
MPAP (mmHg)	14.6±4.7 (7)	15.7±3.6 (7)	16.3±7.1 (7)	17.2±4.0 (6)	0.805
CO (l/min)	2.8±0.5 (6)	2.4±0.6 (7)	2.4±1.0 (7)	2.4±0.9 (5)	0.842
SVR (dyn s cm^{-5})	1,950±318 (6)	2,375±530 (7)	2,452±703 (7)	2,402±679 (5)	0.461
PVR (dyn s cm^{-5})	258±40 (6)	336±122 (7)	390±121 (7)	376±140 (5)	0.208

Data are reported as mean ± SD (number of analyzed values).

CO = cardiac output; HR = heart rate; MAP = mean arterial pressure; MPAP = mean pulmonary arterial pressure; Paw plateau = plateau airway pressure; PVR = pulmonary vascular resistances; R_{intrs} = inspiratory airflow resistance; R_{TOTrs} = inspiratory resistance of the respiratory system; ΔR_{rs} = inspiratory tissue resistance; SVR = systemic vascular resistances.

VAP. Among these pigs, the RML was always affected with an associated coinfection of the right lower lobe in two cases and left upper lobe in one case.

Animals without Oropharyngeal P. aeruginosa *Challenge*

All three animals completed the 72-h study. As shown in figure 6, diverse airway bacterial communities colonized respiratory secretions, at different concentrations. The most common Gram-negative microbes in tracheal secretions were *E. coli* (25% of isolated pathogens) and *B. bronchiseptica* (8.3% of isolated pathogens); whereas, *S. aureus* (19.4% of isolated pathogens) and *S. suis* (19.4% of isolated pathogens) were the most common Gram-positive bacteria (fig. 7). On autopsy, *E. coli* and *B. bronchiseptica* were found in 13 and 20% of the lung tissue samples, respectively. Only one pig developed *B. bronchiseptica* VAP in the RML.

Discussion

In this study, we developed an animal model of VAP, in which the main pathogenic mechanism is through pulmonary aspiration of oropharyngeal secretions colonized by *P. aeruginosa*. The main results of our study are the following: (1) *P. aeruginosa,* instilled into the oropharynx, rapidly translocate into the airways and colonize tracheal secretions; (2) the first signs of infection develop approximately after 48 h of MV; (3) the right medium and lower lobes are the most commonly affected pulmonary sites, which strongly suggests

a gravity-dependent dissemination of the infection; (4) ultimately, VAP is not associated with severe hemodynamic impairment.

Johanson *et al.*[6–8,25,26] developed the first animal model of VAP in primates, which has been crucial in understanding the pathophysiology of the disease and developing preventive strategies. This model closely reflected the human etiology, and the main pathogenic mechanism *via* aspiration across the ETT cuff of colonized oropharyngeal secretions. Nevertheless, the main drawbacks of the model were the scarce animal availability and the high costs of the animals and dedicated settings. Additionally, strict legislations and policies related to the use of research primates make this model impractical nowadays.

In the last two decades, investigators developed alternative VAP models in pigs and sheep. Only one model of VAP associated with tracheobronchial stenosis has been described.[9,10] Interestingly, after injury of the airways, a high incidence of VAP was unintentionally found, mainly caused by endogenous bacteria. This model emphasized the roles of mucus clearance impairment and MV in the development of respiratory infections. The use of this model was marginal, but provided essential insights to develop models in healthy pigs and sheep. Indeed, shortly thereafter Marquette *et al.*[11] demonstrated that the majority of healthy pigs on invasive mechanically ventilation developed VAP within 48 to 72 h. VAP was mainly caused by endogenous oropharyngeal pathogens, such as *P. multocida* and *S. suis*.

Table 2. Bacteriologic Cultures of Tracheal Aspirates and Clinical Pulmonary Infection Score

Pig No.	Hour	Microorganism	Microorganism Concentration (Log cfu/ml)	CPIS
1	0		0.00	1
1	24		0.00	
1	48	Pseudomonas aeruginosa	6.48	
1	72	P. aeruginosa	6.30	6
2	0		0.00	0
2	24	P.aeruginosa	5.92	
2	48	P.aeruginosa	6.77	
2	60	NA	NA	10
3	0		0.00	1
3	24	P.aeruginosa	4.00	
3	48	P. aeruginosa	3.26	
3	48	Bordetella bronchiseptica	3.26	
3	72	P. aeruginosa	6.32	7
4	0		0.00	1
4	24		0.00	
4	48	P. aeruginosa	2.30	
4	48	B. bronchiseptica	2.24	
4	72	P. aeruginosa	6.34	6
5	0		0.00	1
5	24		0.00	
5	48	P. aeruginosa	4.44	
5	48	Escherichia coli	4.00	
5	72	P. aeruginosa	5.93	9
5	72	E. coli	5.00	
6	0	NA	NA	1
6	24		0.00	
6	48	P. aeruginosa	5.76	
6	72	P. aeruginosa	6.98	5
7	0		0.00	1
7	24		0.00	
7	48	P. aeruginosa	5.32	
7	72	P. aeruginosa	6.47	6

cfu = colony-forming unit; CPIS = Clinical Pulmonary Infection Score (see Supplemental Digital Content 2, http://links.lww.com/ALN/B42, which depicts in table 1 the variables to calculate the modified clinical pulmonary infection score); NA = not available.

Fig. 3. Sequential assessment of *Pseudomonas aeruginosa* concentration in tracheal secretions. Data are reported as mean ± SD. Bonferroni-corrected *P* values were obtained by multiplying each *P* value by the number of *post hoc* multiple comparisons. After bacterial challenge, the concentration of *P. aeruginosa* in tracheal secretion progressively increased (*P* < 0.001), reaching 6.39 ± 0.34 log colony-forming unit (cfu)/ml at the end of the study.

Zanella *et al.*[14] found similar results in pigs positioned as in our current study and mechanically ventilated for 72 h. Although these models mimic aspiration of endogenous oropharyngeal pathogens, characteristic human bacteriology is not reproduced. Also, bacterial challenge is not controlled, thus methodological reproducibility is limited and significant discrepancies could occur between subjects or treatment groups. In our model, pulmonary colonization by the most prevalent endogenous pathogens was proficiently controlled with ceftriaxone. Indeed, in our preliminary studies *Pasteurella* and *Streptococcus species* were never found in the lung tissue and VAP hardly developed as previously reported.[11] Also, after bacterial challenge with *P.aeruginosa*, we seldom found endogenous Gram-negative pathogens in tracheal secretio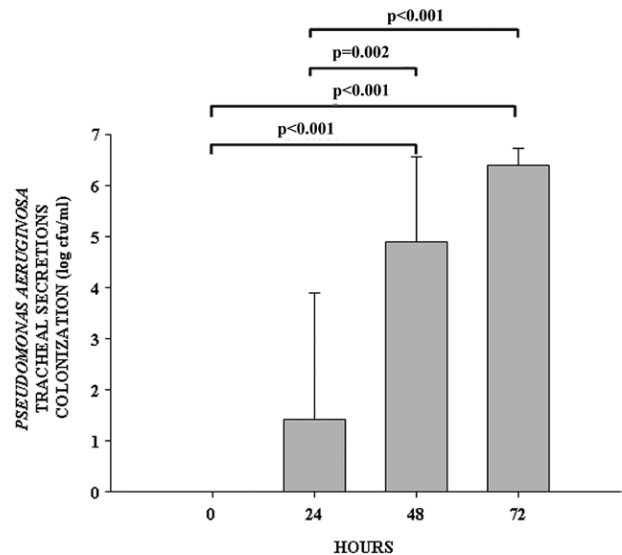ns, that is, *E. coli* and *B. bronchiseptica*. It is likely that the vast inoculum of *P. aeruginosa* could have altered the dynamics of the oropharyngeal and pulmonary microbiota and favored an overgrowth of this exogenous pathogen.

Several reports from the National Institutes of Health[12,13] depicted a VAP model in sheep positioned with a tracheal orientation above horizontal. Similar to the aforementioned models, VAP was caused by endogenous oropharyngeal pathogens, the time to VAP onset was faster than the pig model (approximately 24 h),[12] and the VAP incidence approximated 100%. Nevertheless, sheep are ruminants and opposed to pigs or humans, the large production of oropharyngeal secretions could overestimate risks of pulmonary aspiration across the ETT cuff and lung infections.

Finally, Goldstein *et al.*,[15,16] Luna *et al.*,[17] and Martinez-Olondris *et al.*[18] developed VAP models *via* intrabronchial instillation of nosocomial pathogens. As a result, severe bilateral pneumonia consistently developed after 12 to 24 h, and it was likely intensified by the MV.[27] These models reproduced human etiology, and they have been used to study local and systemic inflammatory responses,[28] as well as effectiveness of antibiotic and novel treatments.[17,29] Yet, in humans, the oropharynx is the primary source of infection[30] and the dependent lung segments are mostly affected.[24] Therefore, these models do not mimic pulmonary aspiration of oropharyngeal pathogens, and the characteristic intrapulmonary distribution of the infection. In our model, the oropharynx was initially colonized, and in 78% of the cases, pneumonia developed in the right medium and lower lobes. A possible explanation
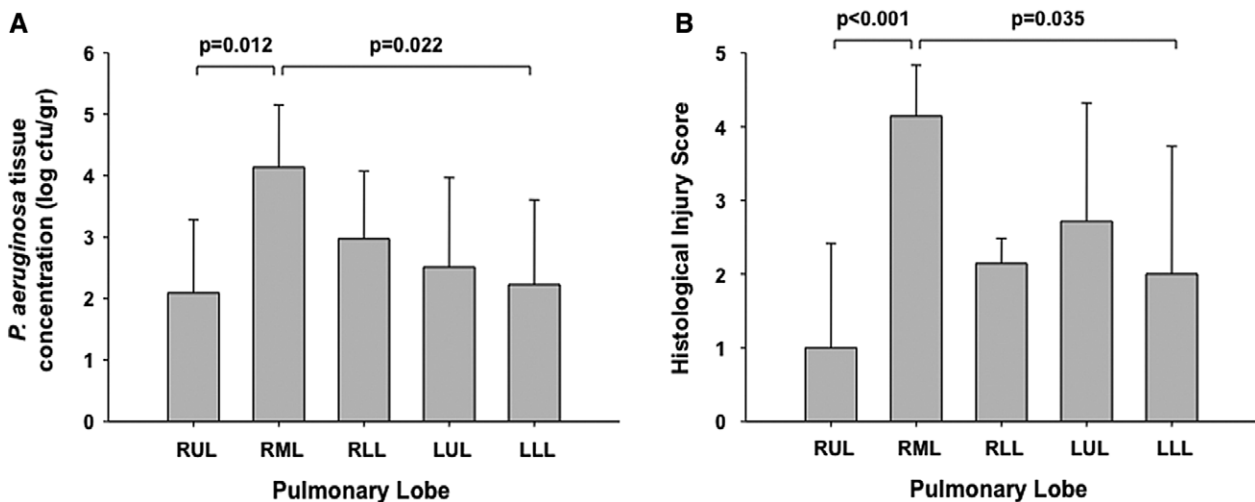
Fig. 4. Microbiological and pathological pulmonary findings, data are reported as mean ± SD. (*A*) Quantitative *Pseudomonas aeruginosa* culture of pulmonary lobes showed significant differences between lobes (restricted maximum likelihood analysis, *P* = 0.009). Thus, the right medium lobe (RML) presented the highest burden, 4.14±1.01 log colony-forming unit (cfu)/g. (*B*) Likewise, pathology studies demonstrated diversity in lobar injury (restricted maximum likelihood analysis, *P* = 0.002). The RML often showed a pattern of confluent pneumonia, mean histologic injury score: 4.14±0.69. Bonferroni-corrected *P* values were obtained by multiplying each *P* value by the number of *post hoc* multiple comparisons. LLL = left lower lobe; LUL = left upper lobe; RLL = right lower lobe; ; RUL = right upper lobe.

of these findings is that pigs were prone, and colonized oropharyngeal fluids could have easily gravitated into the main right bronchus, which is wider and with a less acute angulation in comparison with the left one (fig. 2), and into the RML, which arises from the ventrolateral side of the right bronchus. Additionally, these findings are in agreement with previous postmortem human studies on VAP, which demonstrated a greater incidence of pneumonia in the dependent lung segments and a gravity-dependent dissemination of the infection.[24]
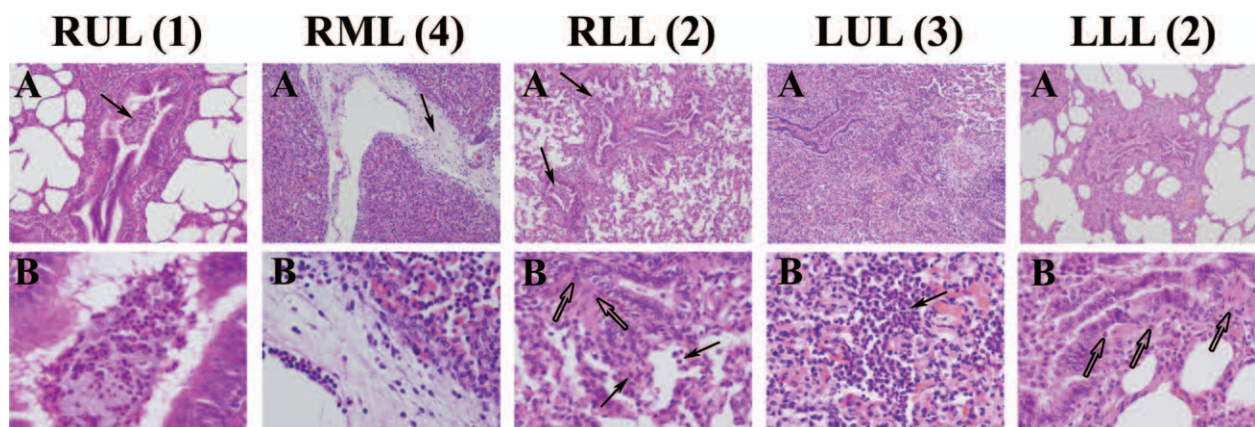

Fig. 5. Characteristic lobar histological pattern. Per each lobe, the number in parentheses is the median infection-related lung injury score. All pulmonary sections were stained with hematoxylin and eosin. Right upper lobe (RUL): (*A*) (×100) a purulent mucus plug is within the bronchiole lumen, indicated by the *filled arrow*. The surrounding alveolar spaces are clear. (*B*) (×400) Polymorphonuclear cells are visible within the mucus plug. Right medium lobe (RML): (*A*) (×100) pneumonia is confluent, as the polymorphonuclear infiltrate is identifiable within three contiguous secondary lobes, separated by a septum (*filled arrow*). (*B*) (×400) Polymorphonuclear cells are seen on both sides of the interlobular septum. Right lower lobe (RLL): (*A*) (×100) the *filled arrows* indicate mucus plugs within the bronchiolar lumens, associated with bronchiolar wall alterations. (*B*) (×400) Polymorphonuclear infiltrate is visible within the bronchiolar wall, specifically, the *emptied arrows* indicate polymorphonuclear cells within the muscle fibers and the *filled arrows* show them extending toward the alveolar spaces. Left upper lobe (LUL): (*A*) (×100), extensive polymorphonuclear infiltrate is found within the alveolar spaces. (*B*) (×400) At higher magnification, the *filled arrow* shows a severe buildup of polymorphonuclear cells. Left lower lobe (LLL): (*A*) (×100) similar to the aforementioned pattern, a purulent mucus plug is seen within the bronchiolar lumen, associated with bronchiolar wall alterations. (*B*) (×400) The *emptied arrows* indicate polymorphonuclear cells within the muscle fibers of the bronchiolar wall. 0, no injury; 1, purulent mucous plugging; 2, bronchiolitis; 3, pneumonia; 4, confluent pneumonia; 5, abscessed pneumonia (cellular necrosis coexisting with disruption of cellular architecture)
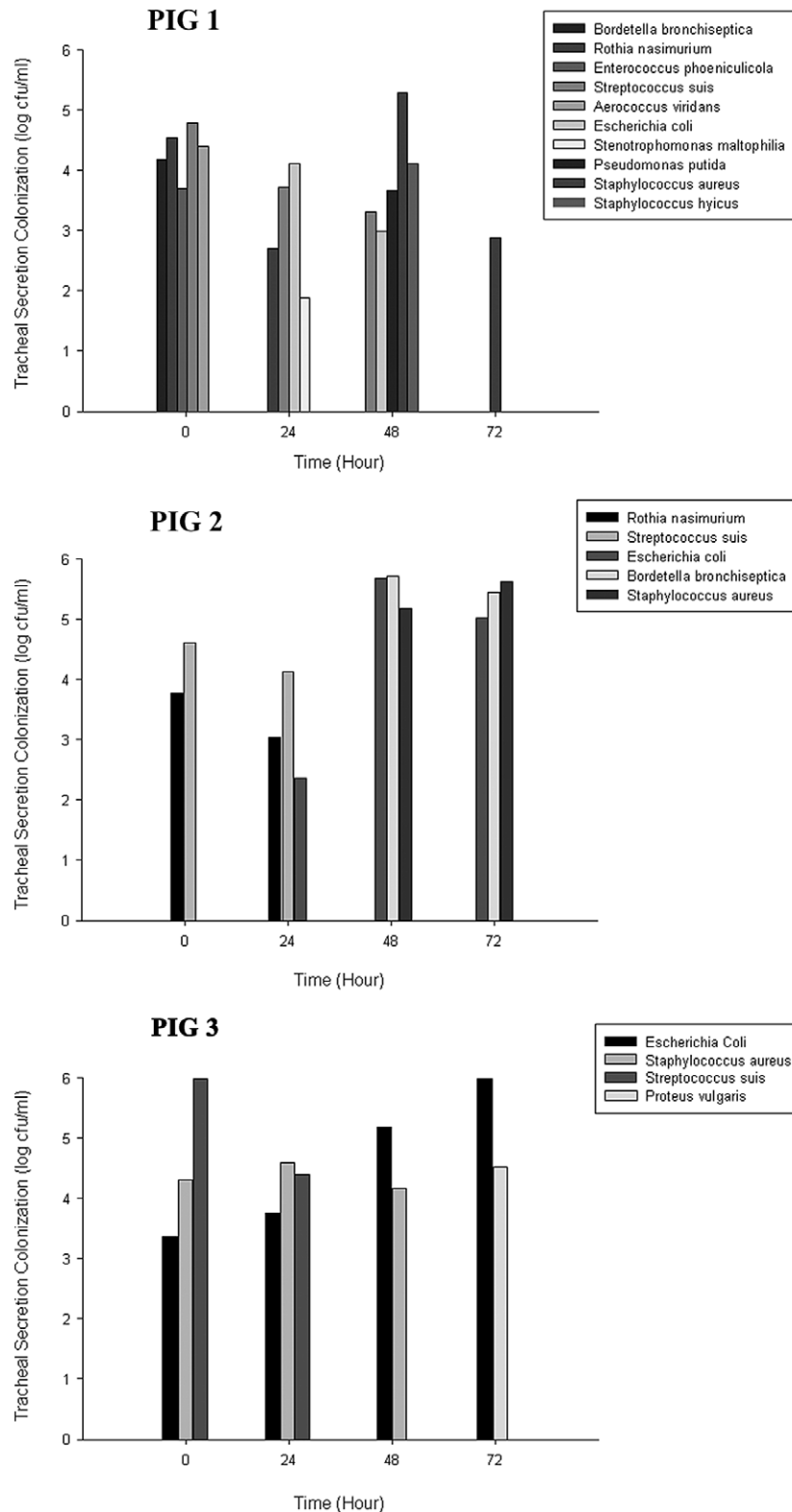
Fig. 6. Sequential assessment of bacteria concentration in tracheal secretions of animals not challenged with *Pseudomonas aeruginosa*. Data are reported as log of colony-forming units per milliliter.

As corroborated by the gradual colonization of respiratory secretions (fig. 3), a continuous microaspiration of colonized oropharyngeal secretions across the cuff likely occurred in our model, regardless of the tight control of the internal cuff pressure. We used an ETT comprising a polyvinyl chloride high-volume low-pressure cuff (Hi-Lo).
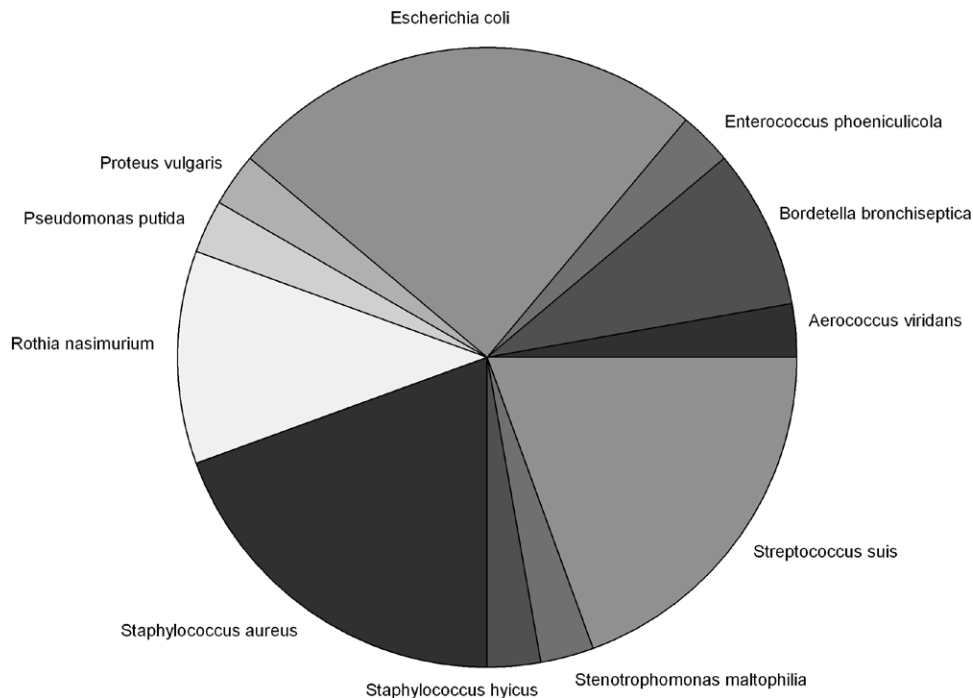
Fig. 7. Tracheal secretions of bacterial diversity in animals not challenged with *Pseudomonas aeruginosa*. Data are reported as percentage of isolated bacteria among tracheal aspirate samples: *Aerococcus viridans*, 2.78%; *Bordetella bronchiseptica*, 8.33%; *Enterococcus phoenicula*, 2.78%; *Escherichia coli*, 25%; *Proteus vulgaris*, 2.78%; *Pseudomonas putida*, 2.78%; *Rothia Nasimurium*, 11.11%; *Staphylococcus aureus*, 19.44%; *Staphylococcus hyicus*, 2.78%; *Stenotrophomonas maltophilia*, 2.78%; *Streptococcus suis*, 19.44%.

This is the most commonly used cuff design in critical care, although several *in vitro*[31–34] and *in vivo* studies[35] have reported limited sealing efficacy. Importantly, we recently corroborated substantial microaspiration across the Hi-Lo cuff while it was inflated at an internal cuff pressure of 30 cm H_2O.[32] When the cuff OD is larger than the tracheal diameter,[31] folds form along the cuff surface and fluids leak through these folds. In our model, the ratio between the ETT cuff OD and tracheal diameter was 1.9; accordingly, several folds may have formed. Additionally, in our model, we did not apply PEEP because laboratory[36,37] and clinical studies[38] have shown reduction of lung infections with its use. As a result, microaspiration of oropharyngeal contents was likely enhanced.[33,34,39]

Our study has several additional features that merit consideration. Previous reports[11] used a quantitative lobar culture of 4 log cfu/g or greater for the diagnosis of pneumonia. Conversely, we used a quantitative *P. aeruginosa* lobar culture of 3 log cfu/g or greater as a threshold for the diagnosis of VAP, because we administered ceftriaxone that may affect cultures of lower airway samplings and decrease diagnostic sensitivity.[40–42] Importantly, the development of pneumonia was associated with clinical signs of infection and the worsening of respiratory parameters, without hemodynamic compromise. These results are consistent with previous reports,[11,17] and underline a compartmentalized rather than systemic inflammatory response of VAP. Importantly, we used a tidal volume of 10 ml/kg and no PEEP. A few reports

in pigs demonstrated that MV without PEEP[27,36] and tidal volumes as low as 6 to 8 ml/kg[43] could potentially result in ventilator-induced lung injury. Therefore, although in our model the plateau airway pressure was always below 13 cm H_2O (table 2), pulmonary injury caused by the mechanical ventilator could have played a confounding additional role on lung colonization and VAP.

Our VAP model has several advantages: reproducibility, ease of induction, it reflects human etiology, and has a consistent time course of infection. Indeed, the first clinical signs of pneumonia appeared approximately after 48 h of MV and pneumonia was consistently confirmed after 72 h. An additional strength of our study was the assessment of the endogenous respiratory colonization in a limited number of animals treated with ceftriaxone and without bacterial challenge. Our model could conveniently be employed to study pathophysiologic mechanisms of the disease and diagnostic strategies and to test the effectiveness and safety of novel strategies aimed at preventing translocation of oropharyngeal pathogens into the lungs. ETTs comprising novel leak-proof cuffs or aspiration of subglottic secretions, as well as new agents for oropharyngeal decontamination, could be tested in this model. Conversely, due to the time required for VAP development, this model is not feasible for pharmacological studies.

A few limitations of our experimental model need to be discussed. First, we studied a small population; nevertheless, our findings were consistent and VAP developed in 85% of

the cases. Second, our results may not be applicable in subjects intubated with different ETTs. In particular, ETTs comprising narrower OD cuffs may reduce aspiration of colonized oropharyngeal secretions. Third, we did not assess biofilm formation within the ETT. Yet, in our model, translocation of pathogens from within the ETT could have played an additional role in the development of VAP.[44] Fourth, the animals were kept prone whereas human patients are normally kept supine. This prone position was necessary because pigs develop diffuse areas of atelectasis when they are supine for a prolonged period of time. Fifth, animals were deeply sedated and paralyzed. In critical settings, a deep sedation/paralysis is only applied to the most critical patients, and during the very first period of MV; interestingly, these patients also present the greatest risk for VAP development.[45] Sixth, pigs were healthy at the time of intubation; hence innate host's respiratory defenses could have been more effective in comparison with that of severely ill patients.

In conclusion, we demonstrated that VAP consistently developed within 72 h after oropharyngeal *P. aeruginosa* challenge in tracheally intubated and mechanically ventilated pigs, positioned in anti-Trendelenburg. The main advantage of this experimental model is that it closely reflects primary pathogenic mechanisms of human VAP and bacteriology. Thus, it could be used to study the pathophysiology of the disease, including diagnostic and preventive strategies.

Acknowledgments

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Torres: Servei de Pneumologia iAl·lèrgiaRespiratòria, Hospital Clínic, Calle Villarroel 170, Esc 6/8 Planta 2, 08036 Barcelona, Spain. atorres@clinic.ub.es or www.idibapsrespiratoryresearch.org. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Infectious Diseases Society of America: Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. Am J Respir Crit Care Med 2005; 171:388–416
2. Ibrahim EH, Tracy L, Hill C, Fraser VJ, Kollef MH: The occurrence of ventilator-associated pneumonia in a community

hospital: Risk factors and clinical outcomes. Chest 2001; 120:555–61
3. Rello J, Ollendorf DA, Oster G, Vera-Llonch M, Bellm L, Redman R, Kollef MH; VAP Outcomes Scientific Advisory Group: Epidemiology and outcomes of ventilator-associated pneumonia in a large US database. Chest 2002; 122:2115–21
4. Warren DK, Shukla SJ, Olsen MA, Kollef MH, Hollenbeak CS, Cox MJ, Cohen MM, Fraser VJ: Outcome and attributable cost of ventilator-associated pneumonia among intensive care unit patients in a suburban medical center. Crit Care Med 2003; 31:1312–7
5. Rouby JJ, Bouhemad B, Monsel A, Brisson H, Arbelot C, Lu Q; Nebulized Antibiotics Study Group: Aerosolized antibiotics for ventilator-associated pneumonia: Lessons from experimental studies. ANESTHESIOLOGY 2012; 117:1364–80
6. Johanson WG Jr, Holcomb JR, Coalson JJ: Experimental diffuse alveolar damage in baboons. Am Rev Respir Dis 1982; 126:142–51
7. Campbell GD, Coalson JJ, Johanson WG Jr: The effect of bacterial superinfection on lung function after diffuse alveolar damage. Am Rev Respir Dis 1984; 129:974–8
8. Crouch TW, Higuchi JH, Coalson JJ, Johanson WG Jr: Pathogenesis and prevention of nosocomial pneumonia in a nonhuman primate model of acute respiratory failure. Am Rev Respir Dis 1984; 130:502–4
9. Marquette CH, Mensier E, Copin MC, Desmidt A, Freitag L, Witt C, Petyt L, Ramon P: Experimental models of tracheobronchial stenoses: A useful tool for evaluating airway stents. Ann Thorac Surg 1995; 60:651–6
10. Marquette CH, Wallet F, Copin MC, Wermert D, Desmidt A, Ramon P, Courcol R, Tonnel AB: Relationship between microbiologic and histologic features in bacterial pneumonia. Am J Respir Crit Care Med 1996; 154(6 Pt 1):1784–7
11. Marquette CH, Wermert D, Wallet F, Copin MC, Tonnel AB: Characterization of an animal model of ventilator-acquired pneumonia. Chest 1999; 115:200–9
12. Li Bassi G, Zanella A, Cressoni M, Stylianou M, Kolobow T: Following tracheal intubation, mucus flow is reversed in the semirecumbent position: Possible role in the pathogenesis of ventilator-associated pneumonia. Crit Care Med 2008; 36:518–25
13. Panigada M, Berra L, Greco G, Stylianou M, Kolobow T: Bacterial colonization of the respiratory tract following tracheal intubation-effect of gravity: An experimental study. Crit Care Med 2003; 31:729–37
14. Zanella A, Cressoni M, Epp M, Hoffmann V, Stylianou M, Kolobow T: Effects of tracheal orientation on development of ventilator-associated pneumonia: An experimental study. Intensive Care Med 2012; 38:677–85
15. Goldstein I, Bughalo MT, Marquette CH, Lenaour G, Lu Q, Rouby JJ; Experimental ICU Study Group: Mechanical ventilation-induced air-space enlargement during experimental pneumonia in piglets. Am J Respir Crit Care Med 2001; 163:958–64
16. Goldstein I, Wallet F, Nicolas-Robin A, Ferrari F, Marquette CH, Rouby JJ: Lung deposition and efficiency of nebulized amikacin during *Escherichia coli* pneumonia in ventilated piglets. Am J Respir Crit Care Med 2002; 166:1375–81
17. Luna CM, Baquero S, Gando S, Patrón JR, Morato JG, Sibila O, Absi R, Famiglietti A, Vay CA, Von Stecher F, Agustí C, Torres A: Experimental severe *Pseudomonas aeruginosa* pneumonia and antibiotic therapy in piglets receiving mechanical ventilation. Chest 2007; 132:523–31
18. Martinez-Olondris P, Sibila O, Agustí C, Rigol M, Soy D, Esquinas C, Piñer R, Luque N, Guerrero L, Quera MÁ, Marco F, de la Bellacasa JP, Ramirez J, Torres A: An experimental model of pneumonia induced by methicillin-resistant *Staphylococcus aureus* in ventilated piglets. Eur Respir J 2010; 36:901–6

19. Guide for the care and use of laboratory animals. Washington, D.C., National Academies Press, 2011

20. Li Bassi G, Saucedo L, Marti JD, Rigol M, Esperatti M, Luque N, Ferrer M, Gabarrus A, Fernandez L, Kolobow T, Torres A: Effects of duty cycle and positive end-expiratory pressure on mucus clearance during mechanical ventilation*. Crit Care Med 2012; 40:895–902

21. Farré R, Rotger M, Ferre M, Torres A, Navajas D: Automatic regulation of the cuff pressure in endotracheally-intubated patients. Eur Respir J 2002; 20:1010–3

22. Martinez-Olondris P, Rigol M, Soy D, Guerrero L, Agusti C, Quera MA, Li Bassi G, Esperatti M, Luque N, Liapikou M, Filella X, Marco F, de la Bellacasa JP, Torres A: Efficacy of linezolid compared to vancomycin in an experimental model of pneumonia induced by methicillin-resistant *Staphylococcus aureus* in ventilated pigs. Crit Care Med 2012; 40:162–8

23. Fartoukh M, Maitre B, Honoré S, Cerf C, Zahar JR, Brun-Buisson C: Diagnosing pneumonia during mechanical ventilation: The clinical pulmonary infection score revisited. Am J Respir Crit Care Med 2003; 168:173–9

24. Rouby JJ, Martin De Lassale E, Poete P, Nicolas MH, Bodin L, Jarlier V, Le Charpentier Y, Grosset J, Viars P: Nosocomial bronchopneumonia in the critically ill. Histologic and bacteriologic aspects. Am Rev Respir Dis 1992; 146:1059–66

25. Johanson WG Jr, Seidenfeld JJ, Gomez P, de los Santos R, Coalson JJ: Bacteriologic diagnosis of nosocomial pneumonia following prolonged mechanical ventilation. Am Rev Respir Dis 1988; 137:259–64

26. Johanson WG Jr, Seidenfeld JJ, de los Santos R, Coalson JJ, Gomez P: Prevention of nosocomial pneumonia using topical and parenteral antimicrobial agents. Am Rev Respir Dis 1988; 137:265–72

27. Sartorius A, Lu Q, Vieira S, Tonnellier M, Lenaour G, Goldstein I, Rouby JJ: Mechanical ventilation and lung infection in the genesis of air-space enlargement. Crit Care 2007; 11:R14

28. Sibila O, Agustí C, Torres A, Baquero S, Gando S, Patrón JR, Morato JG, Goffredo DH, Bassi N, Luna CM: Experimental *Pseudomonas aeruginosa* pneumonia: Evaluation of the associated inflammatory response. Eur Respir J 2007; 30:1167–72

29. Sibila O, Luna CM, Agustí C, Baquero S, Gando S, Patrón JR, Morato JG, Absi R, Bassi N, Torres A: Effects of glucocorticoids in ventilated piglets with severe pneumonia. Eur Respir J 2008; 32:1037–46

30. Feldman C, Kassel M, Cantrell J, Kaka S, Morar R, Goolam Mahomed A, Philips JI: The presence and sequence of endotracheal tube colonization in patients undergoing mechanical ventilation. Eur Respir J 1999; 13:546–51

31. Dullenkopf A, Gerber A, Weiss M: Fluid leakage past tracheal tube cuffs: Evaluation of the new Microcuff endotracheal tube. Intensive Care Med 2003; 29:1849–53

32. Li Bassi G, Ranzani OT, Marti JD, Giunta V, Luque N, Isetta V, Ferrer M, Farre R, Pimentel GL, Torres A: An *in vitro* study to assess determinant features associated with fluid sealing in the design of endotracheal tube cuffs and exerted tracheal pressures. Crit Care Med 2013; 41:518–26

33. Zanella A, Scaravilli V, Isgrò S, Milan M, Cressoni M, Patroniti N, Fumagalli R, Pesenti A: Fluid leakage across tracheal tube cuff, effect of different cuff material, shape, and positive

34. Pitts R, Fisher D, Sulemanji D, Kratohvil J, Jiang Y, Kacmarek R: Variables affecting leakage past endotracheal tube cuffs: A bench study. Intensive Care Med 2010; 36:2066–73

35. Lucangelo U, Zin WA, Antonaglia V, Petrucci L, Viviani M, Buscema G, Borelli M, Berlot G: Effect of positive expiratory pressure and type of tracheal cuff on the incidence of aspiration in mechanically ventilated patients in an intensive care unit. Crit Care Med 2008; 36:409–13

36. van Kaam AH, Lachmann RA, Herting E, De Jaegere A, van Iwaarden F, Noorduyn LA, Kok JH, Haitsma JJ, Lachmann B: Reducing atelectasis attenuates bacterial growth and translocation in experimental pneumonia. Am J Respir Crit Care Med 2004; 169:1046–53

37. Tilson MD, Bunke MC, Smith GJ, Katz J, Cronau L, Barash PG, Baue AE: Quantitative bacteriology and pathology of the lung in experimental *Pseudomonas pneumonia* treated with positive end-expiratory pressure (PEEP). Surgery 1977; 82:133–40

38. Manzano F, Fernández-Mondéjar E, Colmenero M, Poyatos ME, Rivera R, Machado J, Catalán I, Artigas A: Positive-end expiratory pressure reduces incidence of ventilator-associated pneumonia in nonhypoxemic patients. Crit Care Med 2008; 36:2225–31

39. Ouanes I, Lyazidi A, Danin PE, Rana N, Di Bari A, Abroug F, Louis B, Brochard L: Mechanical influences on fluid leakage past the tracheal tube cuff in a benchtop model. Intensive Care Med 2011; 37:695–700

40. Torres A, el-Ebiary M, Padró L, Gonzalez J, de la Bellacasa JP, Ramirez J, Xaubet A, Ferrer M, Rodriguez-Roisin R: Validation of different techniques for the diagnosis of ventilator-associated pneumonia. Comparison with immediate postmortem pulmonary biopsy. Am J Respir Crit Care Med 1994; 149(2 Pt 1):324–31

41. Timsit JF, Misset B, Renaud B, Goldstein FW, Carlet J: Effect of previous antimicrobial therapy on the accuracy of the main procedures used to diagnose nosocomial pneumonia in patients who are using ventilation. Chest 1995; 108:1036–40

42. Souweine B, Veber B, Bedos JP, Gachot B, Dombret MC, Regnier B, Wolff M: Diagnostic accuracy of protected specimen brush and bronchoalveolar lavage in nosocomial pneumonia: impact of previous antimicrobial treatments. Crit Care Med 1998; 26:236–44

43. Ambrosio AM, Luo R, Fantoni DT, Gutierres C, Lu Q, Gu WJ, Otsuki DA, Malbouisson LM, Auler JO Jr, Rouby JJ; Experimental ARDS Study Group: Effects of positive end-expiratory pressure titration and recruitment maneuver on lung inflammation and hyperinflation in experimental acid aspiration-induced lung injury. ANESTHESIOLOGY 2012; 117:1322–34

44. Adair CG, Gorman SP, Feron BM, Byers LM, Jones DS, Goldsmith CE, Moore JE, Kerr JR, Curran MD, Hogg G, Webb CH, McCarthy GJ, Milligan KR: Implications of endotracheal tube biofilm for ventilator-associated pneumonia. Intensive Care Med 1999; 25:1072–6

45. Nseir S, Makris D, Mathieu D, Durocher A, Marquette CH: Intensive care unit-acquired infection as a side effect of sedation. Crit Care 2010; 14:R30