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Pathogenic Link between Post-Extubation Pneumonia and Ventilator Associated Pneumonia: an Experimental Study

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Abstract

Introduction—The presence of an endotracheal tube is the main cause for developing ventilator associated pneumonia (VAP), but pneumonia can still develop in hospitalized patients after endotracheal tube removal (Post-Extubation Pneumonia, PEP). We hypothesized that short-term intubation (24h) can play a role in the pathogenesis of PEP. To test such hypothesis, we initially evaluated the occurrence of lung colonization and VAP in sheep that were intubated, and

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mechanically ventilated for 24h. Subsequently, we assessed the incidence of lung colonization and PEP at 48 h after extubation, in sheep previously ventilated for 24h.

Methods—To simulate intubated ICU patients placed in semi-recumbent position, fourteen sheep were intubated and mechanically ventilated with the head elevated 30° above horizontal. Seven of them were euthanized after 24h (*Control Group*) while the remaining were euthanized after been awoken, extubated and left spontaneously breathing for 48h after extubation (*Awake Group*). Criteria of clinical diagnosis of pneumonia were tested. Microbiological evaluation was performed on autopsy in all sheep.

Results—Only one sheep in the *Control Group* met the criteria of VAP after 24h of mechanical ventilation. However, heavy pathogenic bacteria colonization of trachea, bronchi and lungs (range, 10^4 - 10^9 CFU/g) was reported in four out of seven sheep (57%). In the *Awake Group*, one sheep was diagnosed with VAP and three developed PEP within 48h post extubation (42%), with one euthanized at 30h because of respiratory failure. On autopsy, five sheep (71%) confirmed pathogenic bacterial growth in the lower respiratory tract (range, 10^3 - 10^9 CFU/g).

Discussion—Twenty-four hours of intubation and mechanical ventilation in semi-recumbent position leads to significant pathogenic colonization of the lower airways, which can promote the development of PEP. Strategies directed to prevent pathogenic microbiological colonization before and after mechanical ventilation should be considered in order to avert the onset of PEP.

Keywords

Nosocomial pneumonia; Ventilator associated pneumonia; Post-extubation pneumonia; bacterial colonization

Introduction

Hospital acquired pneumonia (HAP) is still an unresolved issue in Intensive Care Unit.¹ HAP causes clinical outcomes deterioration, death and relevant increase of medical related costs.² VAP is a nosocomial pneumonia that develops in patients intubated and mechanically ventilated for more than 48 hours.³ The major risk factor of VAP is the presence of the **endotracheal tube**,^{4,5} which leads to impairment of tracheal mucus clearance, aerosolization of bacterial biofilm from the endotracheal tube **endotracheal tube** lumen into the lungs^{6,7} and microaspiration of oro-pharyngeal and gastric secretions through the **endotracheal tube** cuff

After extubation, despite **endotracheal tube** removal, the risk of PEP remains still high in both medical or surgical patients.⁸⁻¹⁰ The dynamic of extubation seems to play a relevant role in the new onset of PEP. In a multicenter prospective study on mechanically ventilated patients, De Lassence et al, reported that new onset of nosocomial pneumonia is more frequent and happens significantly earlier when extubation is accidental.¹¹ However, the pathophysiology and the mechanisms behind the development of PEP are still unclear.

In prior studies, we reported that sheep mechanically ventilated for 72 hours or longer with an **endotracheal tube** angle of 30 degree or greater from horizontal always developed VAP in the absence of antibiotics.^{12,13} We demonstrated that bacteria-laden mucus follows

gravity and flows backward from the trachea into the lungs, resulting in lung colonization and pneumonia.¹⁴ In a subsequent study, we further described that pathogenic bacteria heavily colonized the lungs of five out of eight sheep after solely 24h of mechanical ventilation, in absence of any symptom and sign of VAP.¹⁵ Therefore we hypothesized that, in absence of antibiotic prophylaxis, 24 hours of invasive mechanical ventilation in sedated, and paralyzed sheep with head positioned above horizontal might lead to silent bacterial colonization of the lungs, and possibly PEP.

This experimental study was aimed to evaluate such hypothesis and clarify if the lung and lower airways microbiological colonization secondary to 24h mechanical ventilation might lead to a subsequent clinical pneumonia in the 48 hours after extubation.

Methods

This protocol was approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute of the National Institutes of Health, Bethesda, Maryland.¹⁶

We studied 14 young (**2-3 months old**), healthy, female Dorset sheep randomized into two groups:

- 1) *Control Group*: Seven sheep intubated, sedated, paralyzed, and mechanically ventilated for 24h with the head and neck elevated 30 degrees above horizontal to simulate semi-recumbent position of intubated patients in the intensive care unit. Autopsy was performed after 24h of **mechanical ventilation**.
- 2) *Awake Group*: Seven sheep intubated, sedated, paralyzed, mechanically ventilated for 24h, with the head and neck elevated 30 degrees above horizontal (as in the *Control Group*). Sheep were then awakened, maintained at the NIH stable with free access to water and food and euthanized 48h after extubation.

Study design is summarized in Figure 1.

Sheep Preparation, Instrumentation and Measurements

Preparation for the experiment including anesthesia, mechanical ventilation, humidification, feeding of the distal gut without use of prokinetic drugs, and recording of vital signs were obtained as previously described^{12,15} (**please see** Supplemental material). Neither systemic nor topical antibiotics were administered to any sheep in the four weeks prior to the study and during the experiment. Toilette of the mouth and of the perineal area of the animals was carefully provided. Betadine solution was applied daily to surgical incisions. All the circuits used for the ventilator were sterile and equally managed. After extubation, sheep that had been awakened were placed in the NIH stables where the animals had free access to water and food. Clinical assessment along with arterial blood gas analysis and blood chemistry was performed at 24h after mechanical ventilation (both groups) and at 48h after extubation (*Awake Group*) until the end of the experiment. Both groups were euthanized with an intravenous injection of a high dose of sodium pentobarbital and 60 mEq of potassium chloride.

Clinical Criteria of VAP and PEP

During mechanical ventilation, clinical diagnosis of VAP was defined as the occurrence of new pulmonary opacities or lung infiltrates on chest radiograph combined with at least one of the following laboratory criteria: a) white blood cell count $9000/\mu\text{L}$ or $3000/\mu\text{L}$ or b) impairment of gas exchange with a decrease of the arterial oxygen partial pressure/oxygen inspiratory fraction ratio ($P_a\text{O}_2/F_i\text{O}_2$) of at least 30%, as previously described.^{12,14}

After awakening, clinical diagnosis of PEP was proposed in presence of behavioral alterations characterized by slow breathing or tachypnea with cough, or drowsiness, or refusal to eat or drink with at least one of the following radiographic and laboratory criteria: a) new pulmonary opacities or lung infiltrates on chest radiograph, b) white blood cell count $9000/\mu\text{L}$ or $3000/\mu\text{L}$ or c) impairment of gas exchange with a decrease $P_a\text{O}_2/F_i\text{O}_2$ of at least 30%. Fever was not used as diagnostic criteria as a thermal blanket with a rectal probe was used to keep temperature constant during the 24 hours of mechanical ventilation under anesthesia.

Autopsy and Microbiological Sampling, and Blood Cultures

Autopsy and microbiological samplings were performed with strict aseptic technique, as described in previous studies.^{12,13} Gross findings and abnormal lung/body weight ratio ($>0.88\text{--}1.12\%$)¹³ were reported if present at macroscopic exam. On autopsy, 11 samples were taken from the lower respiratory tract: one from the trachea 2 cm above the carina and one from each of the five main bronchi and lobes (Figure 2A). The samples were then sent to the NIH microbiology laboratory to assess the presence of pathogenic bacteria. Bacteria colonization was expressed as colony-forming unit (CFU) over gram in each tissue sample. Descriptive analysis of the microbiological colonization in terms of pathogenic or not pathogenic species was provided between the groups, as previously reported.¹²

Statistical analysis

Normality of data was assessed using the Shapiro-Wilk test. Continuous variables were expressed as mean $\pm$ SD, or range. Statistical significance was reached when the p-value <0.05 (two-tailed). Statistical analyses were performed using STATA-12 (StataCorp LP, College Station, TX, USA) and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel for Mac 2011, Version 14.4.7.

Descriptive characteristics and clinical findings—According to the data distribution, continuous variables were compared with unpaired Student's t-test or Mann–Whitney *U* test between the groups, and with paired Student's t-test or Wilcoxon signed rank test within the groups. Differences over more than 2 timepoints (i.e. Awake Group) was assessed using Friedman test with Dunn's post-hoc analysis.

Microbiological Colonization—Locations were expressed in terms of trachea (T, 1 location), bronchi and lobes (BL, 5 bronchi and 5 lobes, 10 locations) or respiratory system (RS, 5 bronchi, 5 lobes and trachea, 11 locations). Differences in pathogenic bacterial load and number of colonized locations between the groups were tested by location and by bacteria type using a Mann–Whitney *U* test or a Fisher's exact test.

Macroscopy—Presence of gross findings in the two groups was compared with the Fisher's exact test. Average in pathogenic bacterial colonization by location in both groups was depicted using the Karnaugh map. Difference in lung/body weight ratio between the groups was tested using unpaired Student's t-test or Mann–Whitney *U* test, as appropriate.

Results

Study population

We studied 14 young female Dorset sheep (*Control* versus *Awake*, 31.3±4.3 versus 33.4±6.8 kg, *p*=0.49). All sheep were considered healthy and free of disease based on clinical criteria, laboratory data and chest radiographs performed one day prior to the experiment.

Clinical findings

At baseline, there was no difference in PaO₂/FiO₂ among groups (482±73 versus 475±40 mmHg, *Control* versus *Awake*, *p*=0.83). In the *Control Group*, PaO₂/FiO₂ at 24h was not significantly lower than baseline (465±96 versus 482±73 mmHg, *p*=0.81). Similarly, in the *Awake Group*, PaO₂/FiO₂ at 24h was not significantly decreased compared to baseline (443±57 versus 475±40 mmHg, *p*=0.22). However, oxygenation significantly worsened at 72h compared to baseline **in the Awake Group** (PaO₂/FiO₂, 359±134 vs 475±40 mmHg, *p*=0.03). White blood cell count did not differ at baseline between the groups (WBC count, *Control* versus *Awake*, 5.3±1.1 versus 5.6±2 × 10³/μL, *p*=0.77). Description of PaO₂/FiO₂ changes from baseline, WBC count at 24h and 72h, and chest radiographic assessment are reported in Table 1.

VAP was diagnosed in one animal in both groups after 24h of mechanical ventilation (*Control Group*, #40; *Awake Group*, #71).

PEP was diagnosed in two animals at 48h following extubation (#70, #77) and in one animal (#397) at 24h after extubation. Sheep #397 was sacrificed due to worsening of the respiratory insufficiency at 30h after extubation (Table 1).

Microbiological Colonization

The localization of the sampling sites is represented in Figure 2A.

In the *Control Group*, sheep #40 confirmed the clinical diagnosis of VAP with a high rate of bacterial colonization in 10 out of 11 samples (*Pasteurella haemolytica* - range, 7.6×10³–1.5×10⁹ CFU/g).

Despite not presenting criteria of VAP, four other animals (#91, #92, #75, #87) showed pathogenic airway colonization. Trachea was colonized (range, 2.5×10⁵–3.7×10⁸ CFU/g) in four sheep (#40, #92, #75, #87) out of five sheep that showed lung bacterial pathogenic colonization (#40, #91, #92, #75, #87). Bronchi samples (range, 1-4) were colonized by pathogenic bacteria (range, 1-2; range of colonization, 4.0×10³–3.2×10⁹ CFU/g). Lung samples (range, 1-5) were colonized by pathogenic bacteria (range, 1-2; range of colonization, 1.5×10⁴–2.0×10⁹ CFU/g). Bacterial pathogenic colonization of the lower airways matched tracheal bacterial pathogenic colonization in sheep #75, #40 and #87.

Two sheep (#51, #1048) did not present any bacterial colonization (Table 2).

In the *Awake Group*, four animals confirmed the clinical diagnosis of PEP (#397, #70, #77, #71) with consistent lung microbiological colonization. Tracheal sample was colonized by one pathogenic bacterium in each sheep (range, 3.8×10^4 – 1.3×10^7 CFU/g). Bronchi samples (range, 1-5) were colonized by pathogenic bacteria (range, 1-2; range of colonization, 8.0×10^3 – 4.7×10^9 CFU/g). Lung samples (range, 1-4) were colonized by pathogenic bacteria (range, 1-2; range, 2.7×10^4 – 4.6×10^9 CFU/g). Pathogenic bacterial tracheal colonization matched with pathogenic bacterial bronchopulmonary colonization in all sheep but #71.

Three sheep (#388, #80, #74) did not exhibit bacterial colonization. (Table 2)

When we compared the two groups in terms of number of colonized locations (Table 3A) or bacterial load (Table 3B), we did not find any significant difference.

Pathogenic bacterial colonization of the respiratory system is illustrated in the two groups in **Figures 2B** (*Control Group*) and **2C** (*Awake Group*).

Macroscopy

Sheep in the *Awake Group* revealed higher occurrence of gross findings compared to *Control group* (Table 4).

In the *Control Group*, the sheep with clinical diagnosis of VAP at 24h (#40) showed presence of atelectasis in three different lobes (Table 4), where pathogenic bacterial colonization was confirmed (Table 2). We reported in sheep #91 the presence of atelectasis in the RUL, which was colonized by pathogenic bacteria (*Moraxella* sp. and *Pasteurella* sp. in RUL, Table 2). Sheep #1048 showed an abnormal elevated lung/body weight ratio, with neither gross findings nor lung colonization nor clinical diagnosis of pneumonia (Table 4).

In the *Awake Group*, the lung body weight ratio tended to be higher compared to *Control Group* (1.17 ± 0.29 versus 0.95 ± 0.17 , $p=0.10$). All animals presented pulmonary gross findings at autopsy, 48h after extubation ($p=0.02$ versus *Control*). Sheep #71, which developed VAP, matched pulmonary gross findings in three lobes (Table 4) with pathogenic bacterial colonization (Table 2). Of the three sheep that developed PEP (#397, #70 and #77), sheep #70 showed pulmonary gross findings in four lobes colonized by pathogenic bacteria. Left lower lobe (LLL) in sheep #397 and right medium lobe (RML) in sheep #77 were atelectatic, but no pathogenic bacterial colonization was assessed.

Discussion

In this experimental sheep model, we investigated the relationship between oro-tracheal intubation followed by 24 hours of mechanical ventilation without antibiotic prophylaxis, and the development of lung colonization and PEP within 48 hours after **endotracheal tube** removal.

We found that: (I) most animals developed pathogenic bacterial colonization in the lungs in the first 24 hours of mechanical ventilation, but did not meet clinical criteria of VAP; (I)

only two animals met criteria of VAP as early as 24 hours from intubation and mechanical ventilation; (III) PEP was a common finding in the first 48 hours after extubation; and, (IV) clinical pneumonia only developed when pathogenic bacteria were confirmed along the airways at the microbiological evaluation.

Prolonged endotracheal intubation is a known associated risk factor of post-extubation aspiration pneumonia in non-neurologic critical ill patients, as observed by Kim and colleagues.¹⁷ Furthermore, Sands et al have reported the dental plaque as a potential source of VAP in mechanically ventilated patients.¹⁸ However, the bacterial flora of the lower respiratory tract after extubation and the link between VAP and PEP has not been fully investigated.

The present report is the first animal study in medical literature of PEP obtained without bacteria inoculation into the lungs but actually simulating a real scenario of an ICU patient in semi-recumbent position intubated and mechanically ventilated for 24h.

The high degree of bacterial lung colonization, also in absence of any clinical **signs** of infection, revealed by the *Control Group* at the end of 24h of invasive mechanical ventilation, allows us to speculate the presence of a common pathogenetic link between VAP and PEP. We believe that the presence of the **endotracheal tube** is the major determinant of lung infection, during either invasive mechanical ventilation or the first 48 hours following extubation. The colonization of the lower airways is invariably caused by many different factors: the leakage of bacteria-colonized subglottic secretions beyond the endotracheal tube cuff, the impaired mucociliary clearance caused by the presence of the tracheal tube cuff, the suppression of cough reflex and the development of atelectasis.¹⁹⁻²¹ The **endotracheal tube** is considered the main etiopathogenic factor causing VAP. It is well documented that **endotracheal tube** cuffs, also when correctly managed, do not guarantee a perfect sealing of the lower airways^{4,5} and that the orientation of the trachea above horizontal (as in the recumbent position) leads to lung colonization, as described in mechanically ventilated animal models of sheep²⁰, pigs²¹ and monkeys.²²

In our model, we discovered lung colonization in five out seven sheep of the *Control Group* after only 24h of mechanical ventilation. A microbiological diagnosis of pneumonia was confirmed on autopsy in four animals of the *Awake Group*, despite 48h after the **endotracheal tube** removal. In both groups, the sheep with no pathogenic bacterial colonization of the lower airways never proved pathogenic colonization of the trachea. These results suggest that, without direct inoculation of bacteria into healthy lungs and without antibiotic prophylaxis, microbiological lung colonization may develop during a relatively short period of mechanical ventilation (24h) and may persist up to 48h after extubation. Furthermore, the lack of pathogenic bacteria along the lower airways seems always associated with absence of pathogens along the trachea.

The persistence of such a pathogenic bacterial load, as shown by the microbiological evaluation of the tissues in the *Control Group*, can promote the development and the progression of an infective process that can eventually progress in a clinical PEP. These results suggest that clinical pneumonia may develop even many hours after the endotracheal

tube removal, due to the persistency of microbiological colonization of pathogenic bacteria along the lower airways.

Girou and colleagues described that in long-term mechanically ventilated patients in a semirecumbent position and with subglottic suctioning, the rate of microbiological colonization of the oropharyngeal and tracheal secretions, can be as high as 75% already after 1 day of mechanical ventilation.²³ In our animal model, 24h of invasive mechanical ventilation led to tracheal colonization in more than 50% of the animals. This could be favored by lack of administration of any antibiotics and presence of a more consistent oropharyngeal flora in sheep. Furthermore, detection of bacterial colonization has been also made possible by a more invasive tissue sampling compared to standard technique utilized to collect clinical specimens. A higher amount of tissue sampling was available for microbiological evaluation.

In clinical practice, the risk of developing pneumonia in patients intubated and mechanically ventilated persists and is consistent after extubation. Previous investigators studied the occurrence of PEP either in medical or in surgical ICU patients. Thille and colleagues observed an almost equally high occurrence of PEP within 72 hours after planned and unplanned extubation (27% versus 30%).⁸ Frutos-Vivar et al. reported a PEP incidence of 16%, in a large study conducted in 900 medical ICU patients undergoing intubation and mechanical ventilation for more than 48 hours. Furthermore, the authors reported a rate of pneumonia up to 23%, in the subgroup of patients that failed extubation and were subsequently reintubated.⁹ In patients intubated and mechanically ventilated for cardiac surgery, Topal AE and colleagues described a rate of development of a postoperative pneumonia of 13% in a cohort of 162 patients. The authors also reported that according to a univariate analysis, the time spent on mechanical ventilation was significantly longer in patients who developed PEP compared to patients who did not.¹⁰

Our results suggest that prevention of PEP should start before extubation, and strategies implemented to reduce the onset of VAP (i.e., perioperative antibiotic prophylaxis, oral selective decontamination, type of **endotracheal tube**, cPAP, **endotracheal tube** removal with PEEP, etc.)^{4,5,24-29} may also help to reduce the incidence of PEP. Further pre-clinical and clinical studies will be needed to verify whether the prevention of VAP may decrease the occurrence of PEP by averting early lung colonization, or if antibiotic prophylaxis during the first 24-48h after extubation is sufficient to prevent lung infection.

This work has several limitations: tissue histopathology was not analyzed and we did not use temperature as clinical sign to define pneumonia. Considering the propensity of sheep model to develop pneumonia under anesthesia, we used a thermal blanket to maintain the animal's body temperature within a predetermined range. The time of observation after extubation in the *Awake Group* was limited to 48 hours, therefore it is not known if the sheep with pathogenic bacterial colonization will develop a clinical pneumonia at later times.

In conclusion, twenty-four hours of intubation and mechanical ventilation greatly increase the likelihood of developing lung colonization that might later evolve into PEP. The

pathogenesis of VAP and PEP may share some common pathways; hence prevention of PEP should start prematurely before extubation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Study design.

Figure 2.

A) Description of the eleven sampling sites for microbiological evaluation along the respiratory system. Average of pathogenic bacterial colonization (colony forming units/gram) in the Control Group (B) and in the Awake Group (C).

T, trachea; RUB, right upper bronchus; RUL, right upper lobe; RMB, right medium bronchus; RML, right medium lobe; RLB, right lower bronchus; RLL, right lower lobe; LLB, left lower bronchus; LLL, left lower lobe; LUB, left upper bronchus; LUL, left upper lobe.

Table 1

Clinical pneumonia after 24h of MV (VAP) and at 48h after extubation (PEP)

	PaO ₂ /FiO ₂ (%increase)		WBC count (K/ μ L)		Chest X-ray (new pulmonary opacities)			Clinical Pneumonia	
Control group (#ID)	24h after MV		24h after MV		24h after MV			VAP	
91	427 (−12.7)		6.3		No			No	
92	513 (+46.6)		8.4		No			No	
51	533 (−0.9)		5.5		No			No	
75	519 (−8.6)		5		No			No	
40	263 (− 48.3)		3		Yes (RUL)			Yes	
87	520 (+6.6)		10.7		No			No	
1048	480 (+11.9)		3.3		No			No	
Awake group (#ID)	24h after MV	48h after extubation	24h after MV	48h after extubation	24h after MV	48h after extubation	Behavioral changes	VAP	PEP
388	473 (−1)	476 (−0.4)	5.8	9.8	No	No	No	No	No
397*	477 (3.7)	81 (− 82.4)	10.9	n.a.	No	n.a.	Yes	No	Yes
70	377 (−17.3)	452 (−0.9)	8.2	14.2	No	Yes (RML,RUL)	Yes	No	Yes
80	357 (−17.4)	416 (−3.7)	5.1	6.9	No	Yes (RLL)	No	No	No
74	515 (−3.7)	414 (−22.6)	7.5	8.1	No	No	No	No	No
77	462 (−11.8)	327 (− 37.6)	4.9	3.8	No	No	Yes	No	Yes
71	443 (+0.9)	348 (−20.7)	2.5	12.2	Yes (RUL)	Yes (RUL,RML)	Yes	Yes	Yes

Sheep #40, in the Control Group, showed criteria of clinical pneumonia at 24h after intubation and mechanical ventilation (VAP).

Three sheep in the Awake Group (#70, #77 and #71) developed criteria of clinical pneumonia after extubation (PEP).

* In the Awake Group, sheep #397 was suspected for clinical pneumonia. Chest X-ray and WBC count was not performed at 48h after extubation, because the animal was sacrificed at 30h hours after extubation due to severe premature respiratory deterioration. PaO₂/FiO₂ was assessed right before the sacrifice.

In bold, clinical criteria of pneumonia.

Abbreviations: RUL, right upper lobe; RML, right medium lobe; RLL, right lower lobe; WBC, white blood cells; MV, mechanical ventilation; n.a., not applicable.

Table 2

Bacterial colonization (colony-forming units (CFU)/g)

Sheep	Clinical Pneumonia	Species	T	RUB	RUL	RMB	RML	RLB	RLL	LUB	LUL	LLB	LLL
Control group #													
91	No	<i>Mos</i>	-	2.8×10 ⁶	4.1×10 ⁶	-	-	-	-	7.1×10 ⁴	7.1×10 ⁴	-	-
		<i>Pas</i>	-	1.6×10 ⁶	8.2×10 ⁶	1.1×10 ⁴	2.0×10 ⁶	-	-	1.2×10 ⁶	1.2×10 ⁶	-	-
92	No	<i>PaH</i>	-	9.2×10 ⁷	1.6×10 ⁹	-	-	3.6×10 ⁷	2.0×10 ⁹	-	-	2.1×10 ⁶	1.1×10 ⁵
		<i>αHest</i>	3.7×10 ⁸	-	-	-	-	1.1×10 ⁷	5.3×10 ⁸	-	-	-	-
		<i>Pas</i>	2.8×10 ⁸	-	-	-	-	-	-	-	-	-	-
51	No	-	-	-	-	-	-	-	-	-	-	-	-
75	No	<i>Pas</i>	2.2×10 ⁷	-	-	-	-	6.9×10 ⁶	1.4×10 ⁵	-	-	-	-
		<i>αHest</i>	9.3×10 ⁶	-	-	-	-	1.5×10 ⁶	7.8×10 ⁴	-	-	-	-
40	Yes	<i>PaH</i>	2.5×10 ⁵	1.6×10 ⁷	1.5×10 ⁹	2.8×10 ⁷	1.5×10 ⁹	-	5.6×10 ⁴	1.7×10 ⁷	1.2×10 ⁵	7.6×10 ³	1.5×10 ⁴
		<i>αHest</i>	-	-	-	-	-	-	-	-	1.5×10 ⁵	-	-
87	No	<i>PaH</i>	2.8×10 ⁷	3.2×10 ⁹	2.1×10 ⁷	-	-	9.0×10 ⁸	8.4×10 ⁸	8.1×10 ⁴	1.6×10 ⁴	4.6×10 ⁸	2.2×10 ⁵
		<i>KPn</i>	-	-	-	-	-	1.6×10 ⁴	2.4×10 ⁴	-	-	4.0×10 ³	-
1048	No	-	-	-	-	-	-	-	-	-	-	-	-
Awake group #													
388	No	-	-	-	-	-	-	-	-	-	-	-	-
397	Yes	<i>PaH</i>	3.8×10 ⁴	1.3×10 ⁵	2.7×10 ⁴	-	-	-	4.5×10 ⁵	3.7×10 ⁵	-	-	-
70	Yes	<i>PaM</i>	1.3×10 ⁷	1.4×10 ⁷	9.0×10 ⁸	3.7×10 ⁶	1.4×10 ⁹	-	-	-	1.8×10 ⁸	4.7×10 ⁹	1.0×10 ⁵
80	No	-	-	-	-	-	-	-	-	-	-	-	-
74	No	-	-	-	-	-	-	-	-	-	-	-	-
77	Yes	<i>Mos</i>	1.9×10 ⁵	1.7×10 ⁵	-	1.4×10 ⁵	-	5.5×10 ⁵	-	6.0×10 ⁵	-	4.4×10 ⁵	-
		<i>PaM</i>	-	2.6×10 ⁵	1.1×10 ⁸	-	-	-	-	-	-	-	-
		<i>αHest</i>	-	-	2.7×10 ⁸	-	-	-	-	-	-	-	-
71	Yes	<i>Mos</i>	6.3×10 ⁶	-	-	3.7×10 ⁴	-	1.4×10 ⁵	2.4×10 ⁵	5.7×10 ⁵	-	2.5×10 ⁴	-

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Sheep	Clinical Pneumonia	Species	T	RUB	RUL	RMB	RML	RLB	RLL	LUB	LUL	LLB	LLL
		<i>PaM</i>	-	8.0×10^3	4.6×10^9	-	9.9×10^8	-	2.4×10^5	-	-	-	-

Pathogenic bacteria: *Mos*, *Moraxella* sp; *Pla*, *Pasteurella* species; *PaH*, *Pasteurella haemolytica*; *PaM*, *Pasteurella multocida*; *PsA*, *Pseudomonas aeruginosa*; *Eco*, *Escherichia coli*; *KPn*, *Klebsiella pneumoniae*; *Stas*, *Staphylococcus* sp; *Pro*, *Proteus* sp; *Mis*, *Micrococcus* sp; *Ens*, *Enterococcus* sp. *aHest*, *Alpha hemolytic Streptococcus* sp. (not *Streptococcus pneumoniae*). We highlighted in bold all the pathogenic bacteria colonizing the tissue sample. Abbreviations: T, trachea; RUB, right upper bronchus; RUL, right upper lobe; RMB, right medium bronchus; RML, right medium lobe; RLB, right lower bronchus; RLL, right lower lobe; LUB, left upper bronchus; LUL, left upper lobe; LMB, left medium bronchus; LML, left medium lobe; LLB, left lower bronchus; LLL, left lower lobe.

Table 3

A) Differences (p value, two-tailed) in the number of colonized locations on autopsy (either total bacterial species or pathogenic bacterial species) by location between the two groups. **B)** Differences (p value, two-tailed) in the bacterial load on autopsy (either total bacterial species or pathogenic bacterial species) by locations between the two groups. T, trachea; BL, bronchi and lobes; RS, respiratory system.

A				
Group		T, 1 location	BL, 10 locations	RS, 11 locations
Awake vs. Control	All bacteria	1	.39	.41
	Pathogenic bacteria	1	.39	.41
B				
Group		T, 1 location	BL, 10 locations	RS, 11 locations
Awake vs. Control	All bacteria	.45	.25	.23
	Pathogenic bacteria	.45	.23	.20

Table 4

Macroscopy

Sheep	Weight	Lungs	Lungs/ Weight*	Abnormal elevated LBW ratio	Gross findings at autopsy	Clinical Pneumonia
Control group #	(kg)	(g)	(%)	Presence	Description	
91	37	240	0.65	No	Atelectasis of <i>RUL</i>	No
92	35	300	0.86	No	-	No
51	30	320	1.07	No	-	No
75	34	300	0.88	No	-	No
40	31	329	1.06	No	Atelectasis of <i>RUL</i> , <i>RML</i> , <i>LLL</i>	Yes
87	25	245	0.98	No	-	No
1048	27	310	1.15	Yes	-	No
Awake group #	(kg)	(g)	(%)	Presence	Description	
388	45	435	0.97	No	Diffuse congestion	No
397	37	347	0.94	No	Little atelectasis <i>RUL</i> , <i>RLL</i> , <i>LLL</i>	Yes
70	25	431	1.72	Yes	Atelectasis of <i>RUL</i> , <i>RML</i> , <i>LUL</i> and some of <i>LLL</i>	Yes
80	33	405	1.23	Yes	Atelectasis of <i>RLL</i> and <i>RUL</i> . Some congestion of the <i>RLL</i> and <i>LLL</i>	No
74	35	320	0.91	No	Little atelectasis in <i>LUL</i>	No
77	26	295	1.13	Yes	<i>RUL</i> and <i>RML</i> atelectasis.	Yes
71	33	433	1.31	Yes	<i>RUL</i> and <i>RML</i> completely collapsed with many hemorrhagic necrotic areas and abscesses. Little atelectasis of <i>RLL</i>	Yes

Control Group: the sheep that developed a clinical pneumonia (#40) had gross finding on autopsy, with a still normal lung/weight ratio.

Awake Group: four sheep (#70, #80, #77, #71) showed a lung/weight ratio more elevated than normal. Sheep #70, #77 and #71 were suspected of post-extubation clinical pneumonia. The sheep #397, which reported gross findings at autopsy without an abnormal elevated lung/body weight ratio, was prematurely sacrificed at 30 hours after extubation, due to severe clinical deterioration.

* *Normal lung/body weight (LBW) ratio:* median, 1.02; range, 0.88–1.12%.¹³ Abbreviation: *RUL*, right upper lobe; *RLL*, right lower lobe; *RML*, right medium lobe; *LUL*, left upper lobe; *LLL*, left lower lobe.