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# Intra-tracheal amikacin spray delivery in healthy mechanically ventilated piglets

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7 Other authors have no conflict of interest to declare.

8

9

1 **ABSTRACT:**

2 Background: Nebulization during mechanical ventilation is impeded by large extra-pulmonary  
3 drug deposition and long administration durations which currently limit implementation of  
4 inhaled antibiotic therapy. Direct intra-tracheal delivery using a sprayer represents an appealing  
5 alternative investigated in small animal models, but large animal data are lacking.

6 Methods: Amikacin was administered through intravenous infusion (20 mg/kg), nebulization (60  
7 mg/kg) and direct intra-tracheal spray (30 mg/kg) to 10 intubated piglets, in a randomized cross-  
8 over design. Amikacin concentrations were measured in the serum and pulmonary parenchyma.  
9 Anatomic deposition was investigated using immuno-histochemistry.

10 Results: Spray delivery resulted in higher amikacin outputs than nebulization and infusion.  
11 Pulmonary inhaled delivery techniques yielded much higher lung concentrations and much lower  
12 serum concentrations than intravenous infusion. However, unlike nebulization and infusion,  
13 intra-tracheal spray delivery was associated with more than 100- and 1,000-fold variability in lung  
14 concentrations between and within animals. Amikacin specific immuno-histochemistry showed  
15 consistent bronchial and alveolar drug deposition with all modalities.

16 Conclusion: Nebulization remains the most reliable and simple technique to deliver inhaled  
17 amikacin uniformly to the lung during mechanical ventilation. Further development of tracheal  
18 sprays is required to take advantage of potential benefits related to high drug output and low  
19 extra-pulmonary deposition in large animals.

20 **KEYWORDS:** Nebulizer and vaporizer [MeSH], Inhalation [MeSH], Anti-bacterial agents  
21 [MeSH], Aerosols [MeSH], Spray.

22

## 1. INTRODUCTION:

Inhaled antibiotic therapy in mechanically ventilated patients is an appealing therapeutic modality to treat lung infection. Indeed, antibiotics delivered intravenously lead to high serum concentrations with only a minor proportion of the delivered dose actually reaching the lung tissue where infection takes place. Conversely, an inhaled antibiotic dose is totally delivered to the lungs and only marginally absorbed into the systemic circulation [1-5]. However, clinicians face several hurdles when implementing antibiotic aerosol therapy in critically ill patients undergoing mechanical ventilation [6]. In fact, this practice remains infrequent as compared to bronchodilator inhalation in intensive care units [7]. Large size particle deposition in the ventilator circuit, thus not delivered to the patient, represents the first limitation to efficient aerosol delivery. This is particularly true for continuous aerosol generators, which generate great amounts of aerosol during the patients' expiration which are lost in part in the expiratory limb, potentially impeding expiration and inducing ventilator dysfunction [8, 9]. Adapting ventilator settings to increase inspiratory time and positioning nebulizer upstream in the inspiratory limb, at distance of the Y piece can partially limit this aerosol loss [5]. However, beyond the extra-work load of those adjustments requiring specific circuit tubing, long inspiratory times and associated low inspiratory flow reduce the patients' ventilatory assistance and may be poorly tolerated [5]. A straight forward way to overcome expiratory losses of aerosol has been the development of nebulizers synchronized on insufflation. The main limitation of these systems is the direct increase of nebulization duration for a given dose and, for synchronized jet nebulizers, the poor quality of synchronization [5, 10-12].

Direct intra-tracheal drug delivery using sprayers is frequently performed in small animal experiments to test for efficacy and safety of lung delivered drugs [13-19]. Those spray devices can be positioned through the tracheal tube directly at the entrance of the main bronchi, avoiding any circuit drug deposition. Furthermore, as they may be operated during inspiration only, no expiratory loss is expected. Data are lacking in large animals representative of human adult

1 anatomy but are needed to confirm feasibility and quality of lung delivery observed in rodents  
2 [13-19]. In particular, different particle size distributions may jeopardize alveolar parenchymal  
3 deposition.

4 The objective of this study was to measure lung parenchymal drug concentrations after direct  
5 intra-tracheal spray delivery of amikacin compared to standard optimized vibrating mesh  
6 nebulization and intravenous infusion in a large mechanically ventilated animal model.

7

1 **2. MATERIALS AND METHODS:**

2 The experimental protocol was approved by the regional animal experimentation review board  
3 (Comité d'éthique pour l'expérimentation animale Val de Loire, approval number 2012-04-08)  
4 prior to experiments which were carried out in compliance with the ARRIVE guidelines and  
5 European Union directive 2010/63/EU for animal experiments [20, 21]. Eleven Large White  
6 piglets were used for experiments.

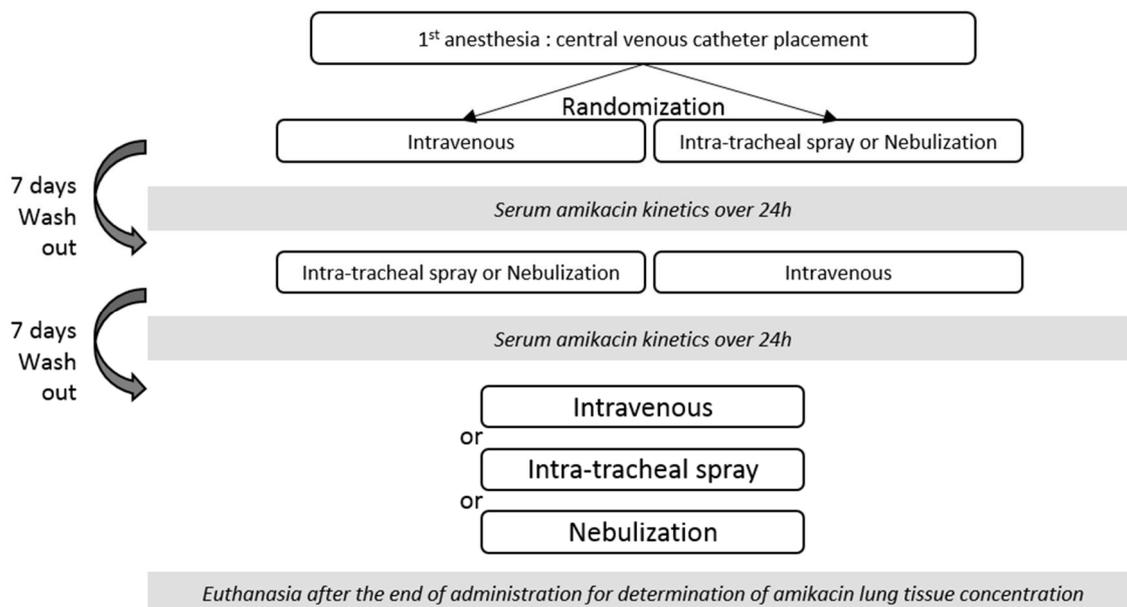
7 *2.1 Experimental design:* For each experimental procedure, piglets were anesthetized with  
8 intramuscular ketamine and xylazine and subsequent isoflurane inhalation, intubated with an oro-  
9 tracheal probe of 6.5 or 7.0 mm diameter and ventilated in volume controlled ventilation with a  
10 tidal volume of 10 mL/kg, a respiratory rate of 15 breath per minute, an inspiratory to expiratory  
11 time ratio of  $\frac{1}{2}$  resulting in an inspiratory flow of 15 L/min, a positive end expiratory pressure of  
12 5 cm of water and an inspired fraction of oxygen of 50%. During a first anesthesia procedure, a  
13 jugular central venous line was placed and subsequently maintained for pharmacokinetic blood  
14 sampling. To compare serum pharmacokinetics of amikacin after intravenous and inhaled  
15 administration to the same animal and measure lung parenchymal concentrations after final  
16 animal euthanasia, a cross over design was used.

17 Animals received amikacin through the intravenous or inhaled route in a computer-generated  
18 random order, implementing a cross-over design with a 7-days washout period (Figure 1).

19

20

1 Figure 1(B&W):

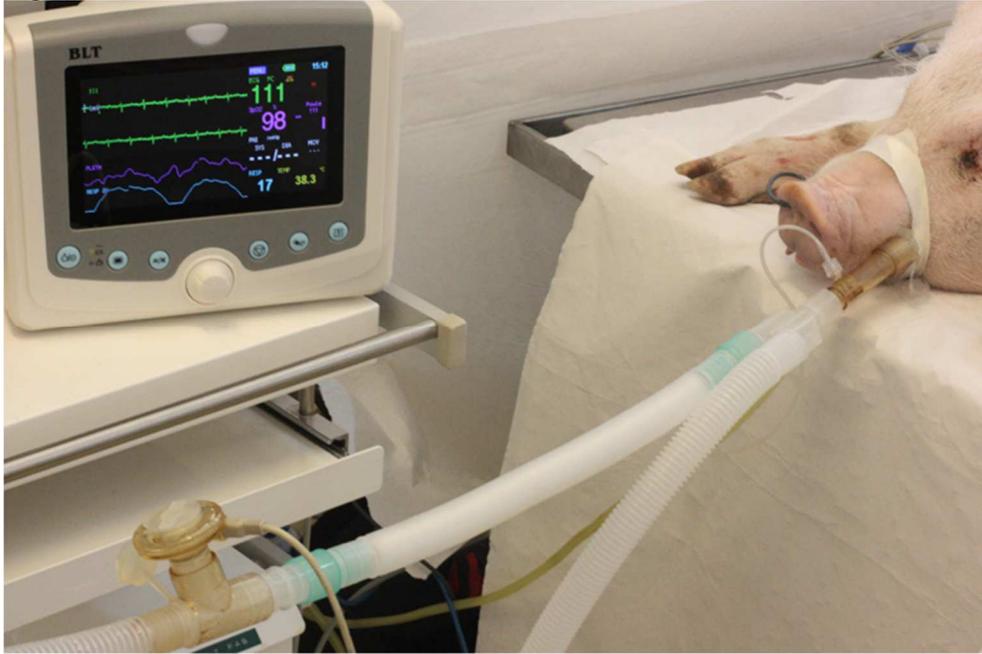


2  
3 Study design: Experiments were performed using a cross-over design, amikacin delivery through  
4 the intravenous route and inhaled route. Inhaled administration was achieved using vibrating  
5 mesh nebulization in one batch of animals and using direct intra-tracheal spray delivery in the  
6 second batch. This design enabled to measure serum pharmacokinetics after nebulization  
7 compared to an intravenous infusions in 5 animals and after intra-tracheal spraying compared to  
8 an intravenous infusion in 5 other animals. Lung parenchymal amikacin concentration was  
9 measured following euthanasia after nebulization in 3 animals, after intra-tracheal spray delivery  
10 in 4 animals and after intravenous infusion in 3 animals.  
11

12 Inhaled administration was achieved using vibrating mesh nebulization within the ventilator  
13 circuit or intra-tracheal spray delivery (n=5 animals for each condition, animal allocation to each  
14 condition was randomly determined). Animals received a third amikacin administration followed  
15 by euthanasia and lung parenchyma sampling (intravenous delivery n=3; vibrating mesh  
16 nebulization n=3; intra-tracheal spray delivery n=4): Figure 1.

17 Intravenous amikacin administration was performed through a peripheral venous line at a dose of  
18 20 mg/kg infused over 1 hour. Nebulization was performed using a vibrating mesh nebulizer  
19 loaded with 60 mg/kg amikacin (1 g in 8 mL), a nominal dose higher than for intravenous  
20 infusion given the anticipated amount of drug deposited in the ventilator circuit and not delivered  
21 to the animal (extra-pulmonary deposition). The nebulizer was operated continuously during the  
22 respiratory cycle (Aerogen Pro®, Aerogen, Galway, Ireland), placed in the inspiratory limb of the  
23 ventilator circuit with a T piece, 20 cm upstream of the Y piece (Figure 2).

1 Figure 2 (Color):



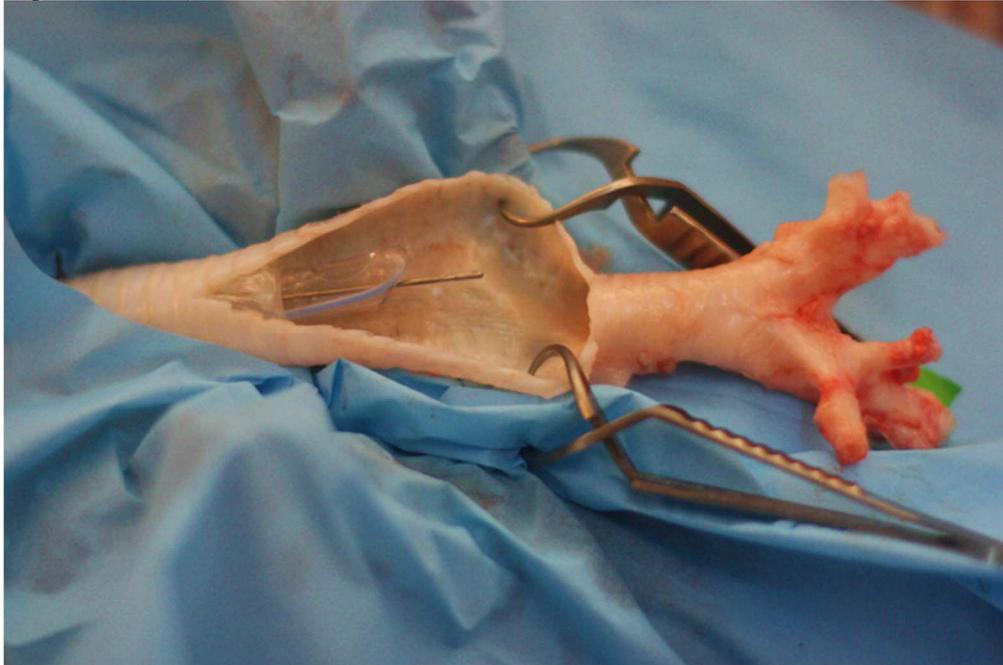
2  
3 The Aerogen Pro® vibrating mesh nebulizer loaded with 60 mg/kg amikacin was placed 20 cm  
4 upstream of the Y piece in the inspiratory limb of the ventilator and operated continuously  
5 during the respiratory cycle.

6  
7 Direct intra-tracheal spray delivery was performed using the MicroSprayer® device (model IA-  
8 1B, Penn-Century, Philadelphia, United States) placed 2 cm distal of the tip of the tracheal tube  
9 under endoscopic visual control (Axess Vision Technology, Tours, France): Figure 3 and Video 1  
10 (online supplement).

11

12

1 Figure 3 (color):



2  
3 Anatomic view of the Microsprayer IA-1B placed 2 cm after the tip of the endotracheal tube (see  
4 online video 1 for *in vivo* endoscopic controlled placement).  
5

6 Thirty mg/kg amikacin (1 g in 8 mL), an intermediate nominal dose due to the anticipated higher  
7 amount of drug delivered to the animal compared to nebulization (reduced extra-pulmonary  
8 deposition) were loaded in the device. Intra-tracheal spraying was synchronized with the ventilator  
9 insufflation phase. The amount of drug delivered at the tip of the endotracheal tube and aerosol  
10 particle size distribution were measured in triplicate *in vitro* prior to experiments (Mastersizer X,  
11 Malvern Instruments, Malvern, United Kingdom). Animals were maintained in prone position  
12 during experiments, and were awakened and extubated at the end of amikacin administration.  
13 After the first two amikacin administrations blood samples were drawn over a period of 24h  
14 (immediate centrifugation and supernatant frozen at -20°C). After the third amikacin  
15 administration animals were maintained anaesthetized and underwent euthanasia by  
16 exsanguination performed immediately after the end of the inhaled administration and one hour  
17 after the end of intravenous administration. Exsanguination was performed by left ventricular  
18 puncture after sternotomy and concomitant infusion of 3-4 liters of sodium chloride 0.9% in the  
19 internal jugular vein to rinse the pulmonary vascular tree and thus avoid any blood contamination

1 when measuring amikacin in the lung tissue. Alveolar lung parenchyma (avoiding large bronchi)  
2 was then sampled in eight regions (upper, middle and lower [2 samples] left lung lobes and  
3 middle and lower [3 samples] right lung lobes) with no additional washing process. The right  
4 upper lobe was purposely not sampled as, unlike in humans, it is directly connected to the trachea  
5 in piglets. Each sample was on one part immediately frozen at -20°C for amikacin concentration  
6 measurement and on the other part fixed in formalin for immuno-histochemistry.

7 *2.2 Amikacin quantification:* Serum amikacin concentrations were determined by enzymatic  
8 immunoassay (EMIT assay Amikacin Cobas+ Mira, Roche Diagnostics, Basel Switzerland). For  
9 lung parenchyma amikacin concentrations measurements, lung sample density was calculated  
10 measuring the mass and volume of the sample before manual grinding in liquid nitrogen and  
11 suspension in 2 mL extraction buffer per gram of lung tissue (Radio-Immuno-Precipitation  
12 Assay: 150 mM sodium chloride, 50 mM Tris-chloride hydroxyde, 1 mM ethylene-diamine-tetra-  
13 acetic acid, Triton 100X 1% sodium deoxycholic acid 1%, 0.1% sodium dodecyl sulfate). Grinded  
14 samples were then homogenized using a disperser and incubated 12 hours at room temperature  
15 followed by 3 hours ultrasonication at 37°C. After centrifugation, amikacin was quantified in the  
16 supernatant, following dilution with commercial serum to meet the validation range of the  
17 technique if necessary (Lyotrol™ N/P, Biomerieux, Marcy l'Etoile, France), using the same  
18 enzymatic technique as for serum analysis. Concentration measurement validation was  
19 established using control lung tissue samples obtained from animals not exposed to amikacin  
20 with exactly the same sampling methodology and spiked with known amounts of amikacin.  
21 Amikacin recovery values were 92.6% and 96.9% for supernatant concentrations of 1.4 and 9  
22 mg/L, respectively, and were within the *a priori* set range of 80-120%. Lower limit of  
23 quantification was 0.5 mg/L. Fidelity values (CV%) were 13.4% and 7.0% for 1.4 and 9 mg/L,  
24 respectively.

1 *2.3 Immunohistochemistry:* after initial histological slide processing (dehydration, paraffin embedding  
2 and microtome slicing) slides were deparaffined and rehydrated, incubated in methanol with 1%  
3 hydrogen peroxide for 15 minutes at room temperature and then saturated with phosphate  
4 buffered saline containing 5% goat serum (Invitrogen life technologies, Paisley, United  
5 Kingdom). Subsequently slides were incubated for 12 hours at 4°C in phosphate buffered saline  
6 containing 2.5% goat serum with a solution of anti-amikacin mouse IgG1 antibody at  
7 concentration of 0.01 mg/mL (AbD Serotec – MCA5729G, Oxford, United Kingdom) or an  
8 isotype control at the same concentration (Vectors Lab, Burlingame, United States). Slides were  
9 then incubated for one hour at room temperature in phosphate buffered saline containing 2.5%  
10 goat serum with an anti-IgG-HRP (horseradish peroxidase) secondary antibody (1/500 dilution,  
11 Jackson Immuno-Research, West Grove, United States). The secondary antibody was revealed  
12 using 3,3 Diaminobenzidine (Liquide DAB Substrate Chromogen System, Dako, Glostrup,  
13 Denmark). Finally, the slides were stained with Harris hematoxylin and observed with a x10  
14 magnitude optical microscope and two representative pictures of each sample were taken: one  
15 centered on the alveolar parenchyma and one centered on a bronchus.

16 *2.4 Statistical analysis:* sample size calculation indicated that with a hypothesis of an area under the  
17 amikacin serum concentration curve of 180 mg.h.L<sup>-1</sup> after intravenous infusion and of 80 mg.h.L<sup>-1</sup>  
18 <sup>1</sup> after intrapulmonary delivery [22], 5 animals are sufficient in the context of a cross-over trial to  
19 detect the difference with a power reaching 100% and an alpha risk of 5%. Eleven animals were  
20 used to account for less favorable pharmacokinetics and potential experiments with incomplete  
21 data. Qualitative variables were expressed as counts (percentage) and compared by the Chi2 or  
22 Fisher exact test as appropriate. Quantitative variables were expressed as median [25<sup>th</sup>; 75<sup>th</sup>  
23 percentile] and compared between groups using the Mann Whitney test or signed rank Wilcoxon  
24 test for unpaired and paired data, respectively. Serum amikacin concentrations were analyzed by  
25 non-compartmental pharmacokinetics. Heterogeneity of amikacin concentrations was empirically  
26 evaluated through computing the ratio between highest and lowest concentrations observed

1 within one animal or in-between animals. Of note, outcome assessments (amikacin concentration  
2 measurement, immunohistochemistry and statistical analysis) were performed blind of treatment  
3 groups. A p value below 0.05 was considered significant.

1 **3. RESULTS:**

2 Preliminary *in vitro* measurements showed that 31% of the Aeroneb Pro® nebulizer charge and  
3 94% of the MicroSprayer® charge were delivered at the tip of the endotracheal tube with a mass  
4 median aerodynamic diameter of respectively 6.3 µm and 37.4 µm. Thus, compared to 20 mg/kg  
5 administered intravenously (100% drug delivery), one can estimate that similar doses have been  
6 delivered to animals via the inhaled route: respectively 19 mg/kg and 28 mg/kg using  
7 nebulization and intra-tracheal spray delivery (Table 1).

8

9

Table 1: Amikacin dose and pharmacokinetics			
	Intravenous infusion	Nebulization	Intra-tracheal spray
Nominal dose	20 mg/kg 694 [610;827] mg	60 mg/kg 1956 [1722;2415] mg	30 mg/kg 1020 [949;1237] mg
Mass Median Aerodynamic diameter	NA	6.3±0.1	37.4±2.0
Faction of particles ≤ 5 µm	NA	36±1.2%	0
Delivered dose	20 mg/kg 694 [610;827] mg (100% of nominal dose)	19 mg/kg 606 [534;749] mg (31% of nominal dose)	28 mg/kg 959 [892; 1163] mg (94% of nominal dose)
Administration duration	60 min	73 min [55 min ; 85 min]	3 [3 ; 3] min
Highest lung parenchyma amikacin concentration	59 mg/kg	634 mg/kg	3195 mg/kg
Lowest lung parenchyma amikacin concentration	1.8 mg/kg	25 mg/kg	2.3 mg/kg
Serum bioavailability	100%	10 [7; 11] %	12 [5; 36] %
Data expressed as mean±SD or median [25 <sup>th</sup> ; 75 <sup>th</sup> percentile]. NA: not applicable			

1

2 In ten animals, amikacin serum concentrations pharmacokinetics could be compared after intravenous and inhaled administration (nebulization and  
3 intra-tracheal spray delivery in, respectively, half of the animals). Lung parenchymal amikacin quantification after euthanasia was performed in 10  
4 animals (one animal died due to anesthesia complication before the third amikacin administration and was replaced): in 3 animals after intravenous  
5 administration, in 3 animals after nebulization and in 4 animals after intra-tracheal spray delivery. Animal weight was 32 [26;41] kg, 35 [30;41] kg and  
6 39 [34;45] kg, respectively, before the first, second and third amikacin administration (usual animal growth). Median nominal doses of amikacin were  
7 694 [610; 827] mg, 1020 [949; 1237] mg and 1956 [1722; 2415] mg for intravenous (20 mg/kg), intra-tracheal spray (30 mg/kg) and nebulization (60  
8 mg/kg), respectively (Table 1). While duration of intravenous infusion was standardized (1 hour), intra-tracheal spraying duration (3 [3; 3] minutes,

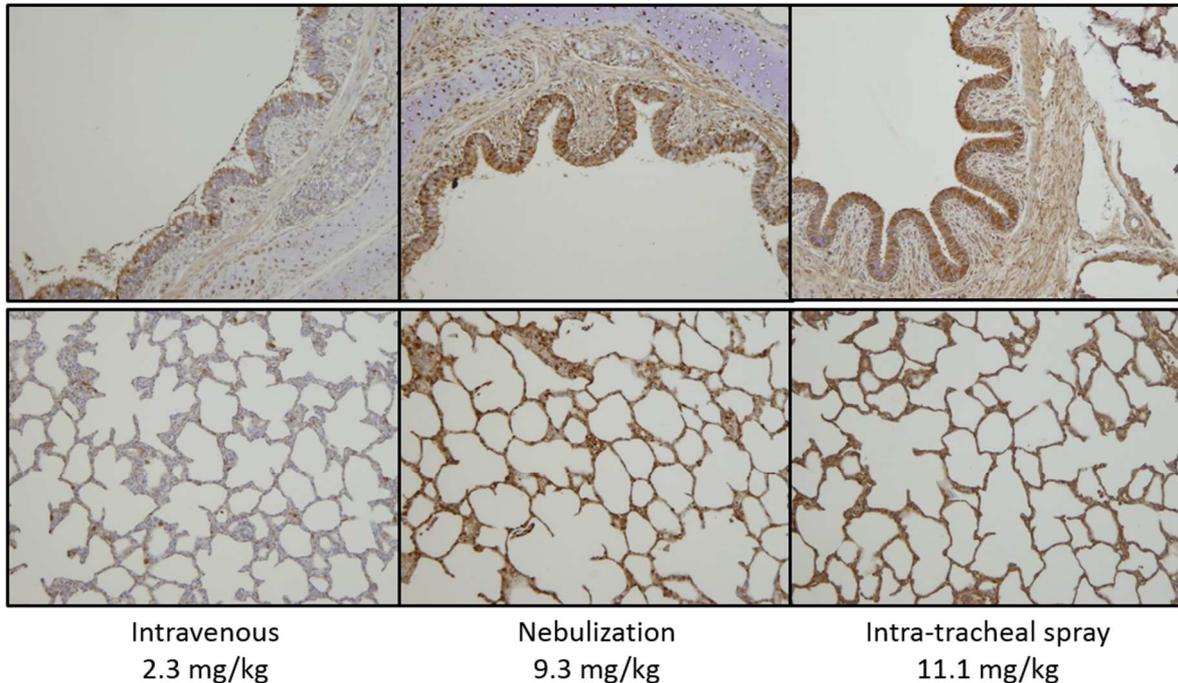
range 2-4 minutes) was significantly shorter than nebulization duration (73 [55; 85] minutes). Corresponding amikacin rates of administration were respectively 367 [327; 445] mg/minute and 27 [24; 29] mg/minute ( $p < 0.01$ ).

Amikacin distribution in lung parenchyma was low after intravenous infusion, with a median concentration of 7.7 [5.9; 11.7] mg/kg of lung tissue. The lowest amikacin concentration measured was 1.8 mg/kg in the left lower lobe of one animal and the highest was 59 mg/kg in the right middle lobe of another animal (ratio 1:33). Within each animal, amikacin concentration ratio between the sample with the highest and the sample with the lowest concentration were 1:1.5, 1:3.1 and 1:13.4 indicating relatively homogenous distribution. Amikacin nebulization within the ventilator circuit was associated with significantly higher overall lung parenchymal concentrations: 184 [49; 296] mg/kg. The lowest concentration measured was 25 mg/kg in the left upper lobe of one animal and the highest was 634 mg/kg in the same left upper lobe of another animal (overall ratio 1:25). Within each animal, the amikacin concentration ratio between the sample with the highest concentration and the one with the lowest were 1:6.9, 1:10.2 and 1:24.0 indicating slightly higher heterogeneity compared to intravenous administration. Intra-tracheal spray delivery was associated with an overall median lung parenchymal amikacin concentration of 10.0 mg/kg, slightly higher than after intravenous administration. However, a great heterogeneity in lung concentrations was observed as the highest concentration was 3,195 mg/kg (right middle lobe of one animal) and the lowest 2.3 mg/kg (left middle lobe of another animal: ratio 1:1,389). Lung parenchymal concentrations 25<sup>th</sup> and 75<sup>th</sup> percentile were 4.0 mg/kg and 161 mg/kg. Heterogeneity was equally high within animals as the amikacin concentration ratio between the sample with the highest concentration and the one with the lowest were 1:2.6, 1:35, 1:75 and 1:140 in the four animals sacrificed after intra-tracheal spray delivery.

For all modes of administration, despite heterogeneity of amikacin distribution within the lung, immunohistochemical analysis showed a consistent bronchial and alveolar site of deposition.

Representative examples of histological slides with significant amikacin deposition are presented in Figure 4.

Figure 4 (color):



Immunohistochemical staining. Photographs at x10 magnification are representative of the total 72 slides analyzed for bronchial and alveolar regions of interest (upper and lower panel respectively). Staining enables qualitative analysis of histological location of anti-amikacin antibodies: beyond bronchial deposition clear alveolar deposition is observed for both inhaled modes of delivery. Amikacin concentrations corresponding to the animal and lung lobe sample pictures are expressed in mg/kg of lung tissue. No staining was observed on negative controls (data not shown).

Beyond qualitative assessment of bronchial and alveolar histological deposition site, immunohistochemistry confirmed heterogeneity of alveolar deposition within and in-between animals.

As expected, serum amikacin concentrations were significantly higher after intravenous administration (peak serum concentration 70 [58; 85] mg/L, area under the serum concentration versus time curve 183 [166; 199] mg.h/L) compared to nebulization (6.0 [5.3; 9.4] mg/L peak serum concentration and 55 [37; 80] mg.h/L area under the serum concentration versus time curve) and intra-tracheal spray delivery (4.8 [1.6; 10.6] mg/L peak serum concentration and 25 [15; 86] mg.h/L area under the serum concentration versus time curve). Bioavailability was

similar after nebulization and intra-tracheal spray delivery: 10 [7; 11] % and 12 [5; 36] % respectively.

#### 4. DISCUSSION:

The main results of this experimental study performed in animals with healthy lungs are that direct intra-tracheal amikacin delivery using a sprayer is feasible in large animals representative of adult human anatomy and enables delivery of large amikacin amounts in a short period of time. High deposition variability represents however a limit of this innovative technique. As for nebulization, amikacin intra-tracheal spray delivery was associated with higher lung parenchymal drug concentrations than intravenous infusion with concomitantly lower systemic exposure. Histologic bronchial and alveolar deposition could be visualized for all modes of administration using drug specific immunohistochemistry. Whereas nebulization duration (median 1 h 13 min) was similar to that of intravenous infusion (1 h), direct intra-tracheal spray delivery allowed a very high amikacin administration rate (367 mg/minute) with a corresponding dosing duration of less than 5 minutes. However, lung distribution of amikacin after intra-tracheal spray delivery was very heterogeneous with concentration variations up to 3,000 fold between animals and up to 140 fold within animals. Vibrating mesh nebulization and intravenous infusion were associated with much more homogenous lung distribution (concentrations varying less than 30 fold between and within animals).

In ventilated piglets with healthy lungs, Goldstein et al. measured lung parenchymal amikacin concentration in the range of 20 to 400 mg/kg of lung tissue after ultrasonic nebulization [23]. The present results show parenchymal concentrations in the same range using a vibrating mesh nebulizer, thus validate the use of the latter aerosol generation technology as well as the parenchymal quantification methodology. In both studies lung parenchymal concentrations were about 10-fold lower after intravenous infusion.

To the best of our knowledge this is the first work investigating direct intra-tracheal spray delivery in large size ventilated animals representative of human adults. We observed a heterogeneous lung distribution of amikacin, in contradiction with several works performed in small rodents that showed homogenous aerosol deposition after spray delivery [14, 15]. Of note,

the variability of amikacin deposition in the lungs after spraying may result in very high local concentration in certain lung areas and raise safety concerns. In a larger sized, non-human primate model, representing a full term newborn (macaque), homogenous deposition was observed using the same device than in the present study [24, 25]. Whereas this observation does not question the relevance of proof of concept studies performed in rodents and small animals in general to investigate inhaled drugs such as antibiotics [13, 17-19], it calls for caution on the translational pathway towards adult human implementation. Differences in animal size in relation to the spray size may in part explain those differences. Indeed, the spray plume at the tip of the sprayer is 10-20 cm long, much larger than the lungs of a small rodent but smaller than the distance needed to reach the extremity of a piglet lung or an adult human lung. Studies on small animals used imaging techniques to quantify aerosol deposition (without regional measurement of actual parenchymal concentrations) and used smaller sized sprayers producing smaller aerosol particles. Conversely, studies including the assessment of parenchymal concentrations in piglets as presently done, did not assess lung deposition using fluorescence or scintigraphy. Studies combining both parenchymal concentration measurement and deposition imaging may give more information in this regard. Actually, such studies, specifically assessing tri-dimensional regional deposition of aerosols coupled to immunohistochemistry and quantification may enable to investigate the apparent paradox of the very large particle size produced by the sprayer (37  $\mu\text{m}$  mass median aerodynamic diameter with no particles smaller than 5  $\mu\text{m}$ ) with concomitant high parenchymal concentrations observed and immunohistochemistry images showing clear alveolar amikacin deposition in our experiments. The standard paradigm linking particle size and proximal to distal lung deposition would indeed predict an exclusively tracheal and large bronchi deposition after intra-tracheal spray delivery. This may be of importance for future aerosol therapy development as intra-tracheal sprayer devices are frequently used to investigate delivery of nanotechnological aerosols [14, 17-19].

The present work presents limitations: bronchial amikacin concentrations were not measured, thus no conclusion can be drawn concerning the value of intra-tracheal spray delivery to target large airways [26]. Different nominal doses used may be seen as a limitation to the comparison of the three modes of delivery. However, the mass of drug administered to the animals were in the range of 500 to 1,000 mg with all modalities and bioavailability was similar between nebulization and intra-tracheal sprayer delivery showing that extrapulmonary drug exposure had been correctly anticipated. Potential differences in drug dose between modalities were several orders of magnitude lower than the differences in amikacin concentrations measured in the lung and serum between techniques and within techniques and animals. Actually, the number of animals may be insufficient to fully investigate regional deposition after intra-tracheal spray delivery given the high variability observed. Great caution was paid when using the intra-tracheal sprayer, in particular, tip placement was visually check using per procedure bronchoscopy to avoid any direct spray on the tracheal wall (Video 1 online supplement) and all possible efforts were made to avoid introducing any air in the system as this may alter aerosol quality. However, some variability observed may be related to the technical implementation of the device.

The present work shows that nebulization remains the most suitable technique to deliver inhaled amikacin to the lung during invasive mechanical ventilation. Despite the clear benefit in terms of amount of administered drug, duration of administration and lack of deposition in the circuit, several factors currently limit implementation of intra-tracheal spray delivery in large animals and thus straightforward human applications: the observed high variability renders difficult to predict efficacy in all lung regions. Some very high local concentrations raise the issue of potential epithelial toxicity. The animals used in the present experiments were healthy, thus, one cannot directly extrapolate results to situations with pathological lungs such as infection or chronic lung disease. Last, the spray procedure may be considered cumbersome to perform. However, the present work, showing feasibility of direct intra-tracheal spray delivery in large animals opens some exciting perspectives such as localized therapy, guiding the device further in the bronchial

tree under bronchoscopic guidance [27]<sup>6</sup>, e.g. localized delivery of biopharmaceuticals using sprayers may enable to precisely target therapy and avoid costly drug losses [28-31]. Developing devices producing even larger particles to avoid alveolar deposition or smaller particles to specifically target alveoli may enable further precision and personalized delivery.

In conclusion, direct intra-tracheal spray delivery of amikacin is feasible in large animals and enables to deliver large amounts of drug, in a synchronized way with insufflation, in a short period of time with no deposition in the circuit. Lung deposition occurred even at the alveolar level despite large particle size distribution of the aerosol. However, unlike in small animals, given the high heterogeneity observed in lung deposition with this new technique, awaiting further investigations, nebulization remains the easiest and most reliable technique to deliver inhaled amikacin uniformly to the lung during invasive mechanical ventilation.

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