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Antimicrobial Coating Prevents Ventilator-Associated Pneumonia in a 72 Hour Large Animal Model

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14. ABSTRACT

Background: Ventilator-associated pneumonia (VAP) affects up to 25% of mechanically ventilated patients with an estimated mortality of 13%. There has been evidence that bacterial biofilms play a role in the development of VAP. Sphingosine-coated endotracheal tubes (ETTs) have demonstrated decreased bacterial adherence to ETT in vitro. Octadecylamine (ODA) is an antimicrobial lipid with a similar structure to sphingosine that has been demonstrated to have broad-spectrum antimicrobial activity. In this study, we hypothesized that ODA and ODA plus doxycycline and levofloxacin-coated (ODA/Abx) ETTs would reduce bacterial adherence within the ETT and lung parenchyma as compared to uncoated ETTs. **Methods:** ETTs were coated with ODA or ODA/Abx using a novel, dip-coating method. Swine were intubated, sedated, and mechanically ventilated for 72 hours using an ODA, ODA/Abx, or uncoated ETT. Blood cultures, arterial blood gas testing, and ETT cultures were performed. Post-mortem histology, lung weight, and bacterial quantification were performed. **Data:** ODA/Abx-coated ETTs significantly reduced bacterial counts in all lobes of the lung and trachea compared to the ODA-coated and uncoated ETT cohorts after 72 hours. Within the distal and proximal ETT, a significant reduction of bacterial counts was seen in the ODA/Abx cohort. In addition, ODA/Abx-coated ETT animals demonstrated significantly less pulmonary edema in the right lung and left lower lung while the ODA-coated ETT induced less edema only in the right lung. **Conclusion:** The current study provides evidence for the application of ODA/Abx coating on ETTs as a novel antimicrobial coating to prevent bacterial colonization on ETTs and in the lungs of swine with a reduction in pulmonary edema. ODA/Abx-coated ETTs may be clinically relevant in the prevention of VAP and associated lung injury.

15. SUBJECT TERMS
Ventilator-associated pneumonia (VAP), endotracheal tubes (ETTs), Octadecylamine (ODA), Sphingosine

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1.0 INTRODUCTION:

Ventilator associated pneumonia (VAP) is one of the most common healthcare associated infections causing significant morbidity and mortality in critically ill patients. It affects 10-25% of mechanically ventilated patients and carries up to a 13% attributable mortality. VAP adds 5-7 days to intensive care unit (ICU) length of stay and increases length of hospitalization by 10-12 days along with its concomitant costs.

VAP is thought to be caused by a variety of factors including the presence of the endotracheal tube, virulence of the offending microorganism, and status of innate immunity. The violation of the natural cough reflex of the glottis due to the presence of the endotracheal tube and the resulting microaspirations around the endotracheal tube cuff are thought to be additional factors. Pathogenic bacteria can then gain access to the lungs either through subglottic secretions, or after having colonized the tube and subsequently pushed through the inspired air via positive pressure ventilation. Colonization of endotracheal tubes also occurs very quickly. There are 106 colony forming units (CFU) of bacteria per centimeter of endotracheal tube after only 24 hours of mechanical ventilation, resulting in a robust biofilm. Development of this biofilm provides a continuous source of potentially pathogenic bacteria invading the lungs continuously.

Multiple methods have been utilized to try to reduce rates of VAP as part of routine ICU respiratory care bundles. While the bundles have provided a modicum of success, the problem persists. Multiple iterations of antimicrobial coated endotracheal tubes have been investigated, including silver sulfadiazine in polyurethane, Silver coated endotracheal tubes have been shown to reduce rates of VAP by 3%, however, their cost has been somewhat prohibitive. Octadecylamine is an antimicrobial lipid found in the skin of humans. It has been studied, along with sphingosine, as a potential factor in the innate immunity against bacterial infection of epithelial tissues. In this study we hypothesized that endotracheal tubes coated with antimicrobial lipids or antimicrobial lipids with antibiotics would significantly reduce colonization of the lungs of pigs with bacteria after undergoing 72 hours of continuous mechanical ventilation compared to uncoated controls. Our primary endpoint was bacterial load of lung tissue. Secondary endpoints included bacterial load of trachea, bacterial load of endotracheal tube segments, survival of pigs.

2.0.Materials and Methods:

2.1.Animal Experiments:

The study protocol was reviewed and approved by the University of Cincinnati Institutional Animal Care and Use Committee (IACUC) and United States Air Force Surgeon General Office of Research Oversight & Compliance. Animals were handled and studies were conducted under a program of animal care accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and in accordance with the "Guide for the

Care and Use of Laboratory Animals" (NRC, 2011; in compliance with DoDI 3216.1)." Pigs were obtained from Isler Genetics (Prospect, OH) with target weights between 60-90 lbs. Trained lab personnel were present with the pigs during the entire 72 hours study duration.

2.2 Preparation of Stock Concentration, and Application of Antimicrobial Molecular Crystal Coating

A concentrated solution of octadecylamine, doxycycline hyclate, levofloxacin, and N-acetylcysteine (ODA + NAC + Abx) was prepared by dissolving solid doxycycline hyclate, levofloxacin, octadecylamine, and N-acetylcysteine in 100% ethanol. The same was completed for octadecylamine and N-acetylcysteine (ODA + NAC) The process involved heating the ethanol to 65°C and slowly adding the solid solute under bath sonication and manual agitation until the resulting solution was transparent. The final concentration of the stock solution was 1.2M octadecylamine, 50 mM doxycycline hyclate, 50 mM levofloxacin, and 72 mM N-acetylcysteine. The specific concentrations were chosen following preliminary experiments. These are the highest reported concentrations of doxycycline hyclate, levofloxacin, and N-acetylcysteine in ethanol. The ODA above its melting point is a miscible liquid with ethanol, however, this concentration is also higher than any previously reported. The stock concentration is then heated to 70°C. Then it is placed in a 250 mL graduated glass cylinder. The endotracheal tubes were warmed to 70°C and straightened by using hemostats clamped to the hard plastic proximal piece. The tubes were immersed into the cylinder slowly and once the tube was completely immersed, it was withdrawn at a rate of 1 cm/sec. They were then allowed to cool at room temperature for 10 min. The tubes were again dipped and cooled again. After they were dry, tubes are then vacuum sealed for storage.

2.3 Intubation, Support Devices, and Mechanical Ventilation

Pigs were initially sedated with a combination of Xylazine (0.1-2 mg/kg), Telazol (4-7 mg/kg), and Atropine (0.04-0.4 mg/kg) intramuscularly. They were then maintained with inhaled isoflurane while support devices were placed. Pigs underwent intubation by direct laryngoscopy using 8.0 endotracheal tubes, either uncoated, or coated. Pigs were then placed in the supine position, and were maintained in this position for the duration of the experiment. Intravenous access with achieved peripherally by cannulation of bilateral marginal ear veins and in some cases central venous access. Continuous blood pressure monitoring was achieved by femoral artery cannulation via a cut down approach. Urinary drainage was achieved via percutaneous catheterization of the bladder. Core temperature was monitored through a rectal probe and temperature was modulated using a heating table and pump system heating pads to maintain a minimum target temperature of 38°C. Continuous peripheral oxygenation monitoring was achieved using an SpO2 monitor placed between the nares. Continuous cardiac telemetry was monitored through subdermal needle electrodes placed on the chest. Mechanical ventilation was continued for up to 72 hours using volume-controlled ventilation (tidal volume, 8–10 ml/kg; respiratory rate, 14–25 breaths/min, fraction of inspired oxygen [FIO_2], 0.4).

2.4 Sedation, IV hydration, Chemical and Ventilator Interventions

Sedation for the 72 hour experimental time course was achieved using a multimodal approach. Inhaled isoflurane (0.5-3%) was used as the initial anesthetic and analgesic. This was augmented with infusion of midazolam (0.4-1 mg/kg/hr) and fentanyl (3-5 µg/kg/hr). However, the porcine resistance to the effects of midazolam was high and its primary use was to allow a moderate titration of isoflurane. Intravascular hydration was maintained with infusion of 5% dextrose containing fluids, primarily 0.9% sodium chloride at a rate ranging from 50 mL/hr to 150 ml/hr with intermittent bolus dosing of 250 – 500 mL to maintain a mean arterial pressure greater than 50 mm Hg. The addition of 50 mEq of sodium bicarbonate was added to each liter of fluids in later experiments as this was discovered to better modulate serum potassium, which had a tendency to become elevated after 24-48 hours of sedation and ventilation. Atropine (0.02 mg/kg) was administered for bradycardia (heart rate < 50). Sodium bicarbonate (50 mEq) was administered for metabolic acidosis (pH < 7.2) or hyperkalemia (potassium > 6.5 mEq/L). Hypoxia was managed by increasing the FiO2 from a starting point of 40% up to 100%. High pulmonary pressure alarms were managed with intermittent endotracheal tube suctioning using a suction catheter, however, this was a rare instance occurring approximately 1 time per 72 hour experiment. Hypercapnia and respiratory acidosis were managed by increasing the respiratory rate and/or tidal volumes to target normal pH.

2.5 Euthanasia and Tissue Harvest

If the pigs were approaching hypoxic respiratory failure, the most common reason for animals dying prior to completion of the 72 hour study, then they were euthanized with Euthasol (pentobarbital, > 150 mg/kg) once they began to have severe cardiac arrhythmias or severe hypotension not responsive to fluid administration. If the pigs completed the 72 hour experiment, they were euthanized in an identical manner.

Samples obtained during the 72 hour experiment included qualitative blood cultures, qualitative endotracheal tube swabs, and arterial blood gas measurements at the start of the experiment immediately after intubation, and then every 8 hours and at the time of euthanasia. In order to harvest lungs and trachea immediately after euthanasia, the pigs' chest was sterilized with chlorhexidine and a median sternotomy was performed using sterilized instruments. The pleura was incised bilaterally and the hilum clamped. The right and left lungs were then removed and placed in a sterile grossing area. The remaining bronchi and trachea were mobilized. The trachea was then incised longitudinally and the endotracheal tube cut at the most cephalad point. Luminal swabs were taken with cotton-tipped applicators and placed into 10 mL of sterile 1X PBS.

2.6 Qualitative blood culture, Qualitative endotracheal tube swabs, and arterial blood gas measurements.

One mL of blood was drawn from the arterial line and plated onto blood agar plates. Sterile cotton tipped swabs were used to swab the proximal 2-3 cm of endotracheal tube immediately distal to the ventilator circuit. These swabs were then plated onto blood agar plates. Plates were incubated

overnight at 37°C and photos taken for documentation. Arterial blood gas measurements were obtained

2.7 Materials

Standard human use endotracheal tubes were obtained from Cardinal Health.

2.8 *Statistics*

Bacterial counts did not follow a normal distribution. Thus, a non-parametric one-way analysis of variance, Kruskal-Wallis test was used to determine statistical significance. Dunn's multiple comparisons was then used to compare treatment groups to control. The reported P values are the adjusted P values from Dunn's multiple comparisons. Figures were plotted as median values with error bars representing interquartile range. Lung mass did follow a normal distribution. An ordinary one-way ANOVA was used to determine statistical significance and a Dunnett's multiple comparison analysis was used to compare treatment groups to control. Figure 2 was plotted as mean with error bars representing standard deviation. A Log-rank, Mantel-Cox, test was used to determine a statistically significant survival difference among groups.

2.9 Power Analysis

The primary endpoint in our study was lung bacterial counts. In order to perform an a priori power analysis for a two-sided t-test, we expected a 50% reduction of bacteria in the lungs of pigs intubated with coated tubes compared to pigs intubated with uncoated tubes. We anticipated a standard deviation of 35% of the mean of the controls. We accepted an alpha = 0.05 for the probability of committing a Type I error and a beta = 0.2 for the probability of committing a Type II error (Power of 0.8). With these parameters, we required a sample size of 6 pigs per group.

3.0 RESULTS:

3.1 ODA + NAC and ODA + NAC + Abx coated ETTs Reduce the Amount of Bacteria Found in Lung Tissue After Prolonged Mechanical Ventilation

Sections of peripheral lung were cut from the right upper lobe (RUL), right lower lobe (RLL), left upper lobe (LUL), and left lower lobe (LLL) and subsequently weighed at the time of pig death or after euthanizing at 72 hours. The amount of bacteria in CFUs/gram of lung tissue was then determined by homogenization and plate dilution outlined above. ODA + NAC + Abx coated tubes significantly reduced lung bacterial infection in the RUL, RLL, LUL, and LLL by 3.7 ($p < 0.0001$), 3.7 ($p < 0.0001$), 3.2 ($p < 0.0001$), and 3.5 ($p < 0.0001$) log, respectively, compared to uncoated control tubes (n = 6 pigs per group). ODA + NAC reduced lung bacterial infection in the RUL, RLL, LUL, and LLL by 2.7 ($p = 0.0035$), 2.3 ($p = 0.0002$), 1.9 ($p = 0.0014$), and 2.3 ($p = 0.0002$) log, respectively, compared to uncoated control tubes (n = 6 pigs per group). (Figure 1)

3.2 ODA + NAC + Abx coated ETTs Reduce the Amount of Bacteria Found in the Trachea After Prolonged Mechanical Ventilation

Segments of trachea were harvested and subsequently weighed at the time of pig death or after euthanizing at 72 hours. The amount of bacteria in CFU/gram of trachea was determined by sonication release of bacteria and plate dilution outlined above. ODA + NAC + Abx coated tubes significantly reduced bacterial colonization of the trachea by 3.1 ($p < 0.0001$) log ($n = 6$ pigs per group) compared to uncoated controls. Notably, ODA + NAC coated ETTs did not reduce bacterial colonization of the trachea. (Figure 1)

3.3 ODA + NAC + Abx coated ETTs Reduce the Amount of Bacteria Found on the ETTs After Prolonged Mechanical Ventilation

Sections of endotracheal tube (1 cm) were serially cut from above the balloon (distal ETT) and from the 22-25 cm markings on the endotracheal tube (proximal ETT) at the time of pig death or after euthanizing at 72 hours. Additional samples of the inner lumen were obtained from 3 cm above the balloon using sterile cotton tipped swabs. The amount of bacteria in CFU/cm tube or CFU per swab were determined by sonication release of bacteria and plate dilution outlined above. ODA + NAC + Abx coated tubes significantly reduced bacterial colonization of the distal ETT, proximal ETT, and inner lumen by 6.0 ($p < 0.0001$) log, 2.8 ($p < 0.0001$), and 6.0 ($p < 0.0001$) log, respectively, compared to uncoated control tubes ($n = 6$ pigs per group). ODA + NAC coated ETTs significantly reduced colonization of the inner lumen by 2.1 ($p = 0.0205$) log, but did not significantly reduce colonization of the total distal or proximal ETT. (Figure 1)

3.4 ODA + NAC and ODA + NAC + Abx coated ETTs were Associated with Lower Combined Lung Mass After Prolonged Mechanical Ventilation

The right and left lungs were weighed immediately after removal at the time of pig death or after euthanizing at 72 hours. The combined mass of the right and left lung was then determined. The combined mass of the lungs from pigs ventilated through ODA + NAC and the ODA + NAC + Abx coated ETTs were significantly less, 34% ($p = 0.0002$) and 23% ($p = 0.0007$), respectively, compared to the combined mass of the lungs removed from pigs ventilated through uncoated control tubes ($n = 6$ pigs per group). (Figure 2)

3.5 ODA + NAC and ODA + NAC + Abx coated ETTs were Associated with Increased Survival of Pigs Subjected to Prolonged Mechanical Ventilation

The study protocol was intended to subject pigs to continuous mechanical ventilation for 72 hours. This duration of mechanical ventilation in pigs has not been described previously. Thus, we found that pigs mechanically ventilated through uncoated control tubes, did not survive to the study completion (median survival 45.3 hours). However, pigs mechanically ventilated through tubes coated with ODA + NAC and ODA + NAC + Abx had a significantly longer ($p = 0.0097$) median survival (70.5 and 71.25 hours, respectively). (Figure 3)

3.6 ODA + NAC and ODA + NAC + Abx coated ETTs Prevent Pulmonary Hepatization and Alveolar Infiltration After Prolonged Mechanical Ventilation

Lungs were immediately harvested at the time of death or euthanization after 72 hours. Prior to gross dissection, photos were taken of the lungs to document their gross appearance (Figure 4). Tissue was then fixed for histology as described above (Figure 5). Lungs from pigs ventilated with uncoated tubes had a more solid appearance, similar to liver tissue, whereas lungs from pigs ventilated with ODA + NAC and ODA + NAC + Abx coated tubes are closer to normal lungs in appearance. (Figure 4) This was further confirmed on H&E histology. Lungs from pigs ventilated with uncoated tubes show hepatization with RBC and fluid infiltration of the alveoli. Whereas alveoli from the lungs of pigs ventilated with coated tubes appear normal. (Figure 5)

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4.0 DISCUSSION:

Our data show that ODA + NAC and ODA + NAC + Abx coated endotracheal tubes reduce the bacterial colonization and infection of peripheral airways in lungs of mechanically ventilated pigs and prevent progressive edema and hepatization of the lungs, resulting in an increase in survival time. This study also shows that ODA + NAC + Abx coated tubes completely prevented accumulation of bacterial biofilm on endotracheal tubes during our study time period.

Our model is the first study to continuously mechanically ventilate pigs for up to 72 hours. Previous studies have ventilated dogs for similar periods of time and sheep for up to 48 hours. Importantly, our study did not employ inoculation with exogenous pathogenic bacteria. Our study relied upon the same mechanisms observed in ventilator associated pneumonia in humans, i.e. colonization of the endotracheal tube and airway leading to infection of the lungs. Our primary endpoint for our study was bacterial counts in the lungs and on the endotracheal tubes. However, upon completion of our study, we found that there was also a statistically significant survival advantage from using the coated tubes (either ODA + NAC + Abx or ODA + NAC) compared to uncoated controls. Pigs have a profound mucus production during mechanical ventilation, which serves as a medium for bacterial growth and subsequent infection. The specific strains of bacteria were not studied, however, samples from each pig were obtained and could be investigated in the future.

The differences between the ODA + NAC and ODA + NAC + Abx groups are notable. Both groups provided a survival advantage compared to uncoated controls. Both groups showed decreased bacterial loads in the lungs. However, the ODA + NAC coated tubes did not provide the same antimicrobial protection against colonization of the endotracheal tubes. N-acetylcysteine was present in both coatings. This was one confounding variable which was not controlled in the study design. In order to design a clinically relevant experiment for large animal mechanical ventilation in an attempt to study ventilator associated pneumonia, the longer the study time, the more relevant the data. This is primarily due to the accumulation of mucus. Thus, the decision was made to add the NAC to the coatings in order to decrease the buildup of frank mucus. The presence of NAC in both ODA + NAC and ODA + NAC + Abx is a potential explanation for the lung bacterial counts and survival advantage of the coated tubes compared to uncoated controls. Thus, further study is

necessary to elucidate whether the ODA or ODA + Abx played a role independent of the NAC in the effectiveness of the coating at decreasing lung bacterial counts and improving survival.

5.0 CONCLUSIONS:

Endotracheal tubes coated with antimicrobial lipids, mucolytics, and antibiotics have the ability to decrease bacterial biofilm during prolonged mechanical ventilation. Combination coatings also reduce bacterial colonization and infection of lungs during mechanical ventilation resulting in a clinically significant improvement compared to uncoated tubes. Determining the role of mucolytics compared to the antimicrobial lipid and antibiotics will require further study.

6.0 Figures:

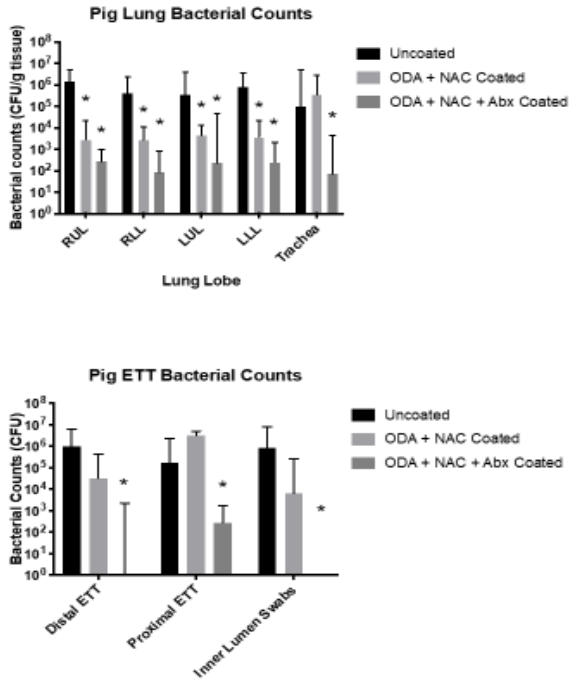

Fig 1: Bacterial Counts

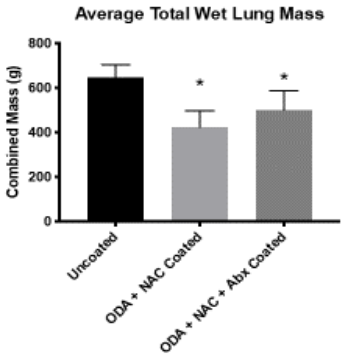

Fig 2: Lung Mass

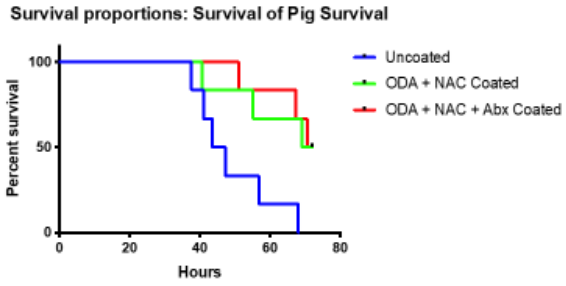

Fig 3: Survival

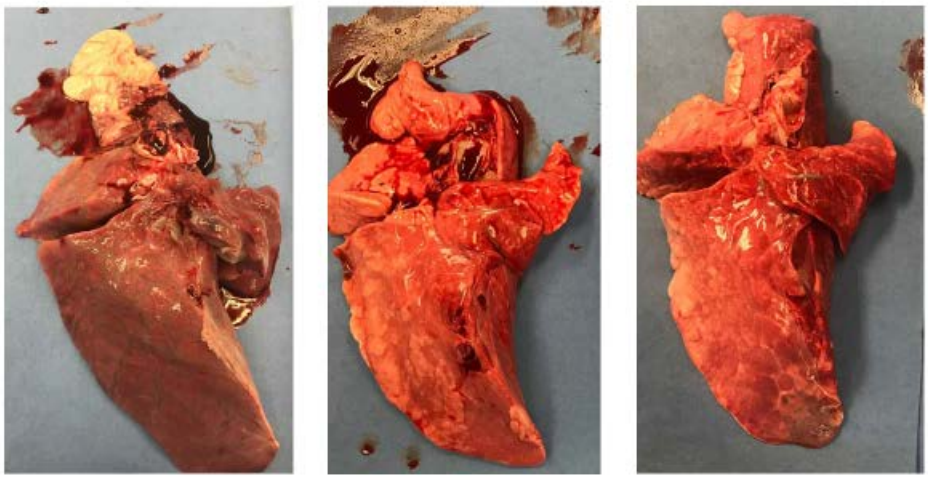

Fig 4: Lung Comparisons

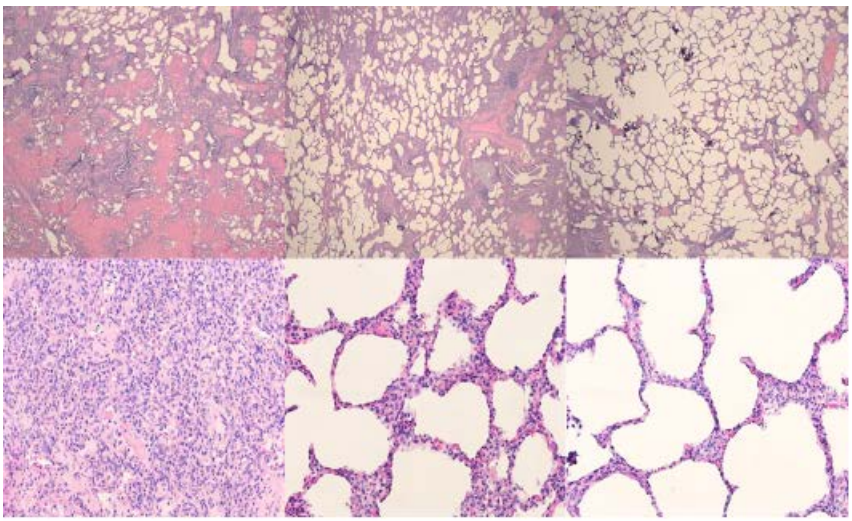

Fig 5: H&E histology