Identification of asinine gamma herpesviruses in a donkey with interstitial pulmonary fibrosis, pleural effusion and thrombocytopenia

**Case Report**

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**Abstract**

A 23-year-old domestic donkey (*Equus asinus*) referred for severe respiratory distress due to suspected equine asthma. Ultrasound of the chest revealed bilateral irregular pulmonary consolidation and pleural effusion. Airway endoscopy and tracheal wash cytology showed severe neutrophilic inflammation and bacterial culture was positive for *Streptococcus equi subsp. zooepidemicus.* Despite aggressive treatment, the donkey died in 48 hours. On post-mortem examination, the lung was whitish, collapsed, and firm, with fibrotic multifocal nodular areas. Pleural effusion and pleuritis were detected. Histologically, the lung architecture was markedly replaced by interstitial fibrosis. The histological features observed were suggestive of a severe chronic fibrosing interstitial pleuropneumonia with type 2 pneumocyte hyperplasia and intralesional syncytial cells. Pulmonary fibrosis was associated with the presence of asinine gammaherpesvirus 2 and 5 infection, confirmed by PCR and sequence analysis. The macroscopic and histological pattern of fibrosis was diffuse and interstitial, and the nodular lesions were consistent with spared lung parenchyma, instead of the canonical nodular distribution of the fibrosis observed in equine

multinodular pulmonary fibrosis. Asinine herpesviral pulmonary fibrosis is uncommon, but should be considered by clinicians in the list of differentials in donkeys with chronic respiratory signs.

**Keywords:** asinine gammaherpesvirus, chronic lung disease, equid, histopathology, interstitial pneumonia, Italy, thrombocytopenia

## Introduction

Pulmonary fibrosis, also known as interstitial fibrosing pneumonia is a severe, uncommon fibrotic lung disease of geriatric donkeys characterized by loss of pulmonary function [1,2] and marked syncytial cell formation [3,4]. Pulmonary fibrosis presents an insidious diagnostic-therapeutic course in geriatric donkeys [3,5,6]. Clinical signs may only become evident in the terminal stage of the disease, when severe dyspnea appears [5,7]. Discriminating pulmonary fibrosis from other causes of chronic lower airway disease, such as severe equine asthma represents a challenge for equine practitioners. Data concerning clinical presentation and diagnostic evaluations, including imaging and tracheal/bronchoalveolar fluid analysis are limited [7]. On post-mortem examination, fibrosis of subpleural and interstitial lung tissues have been occasionally reported in donkeys [5].

Gammaherpesviruses (GHVs) are suspected to be involved as viral pathogens in progressive fibrosing lung disease in humans, rodents, canines, felines, horses, and other equids such as Namibian mountain zebras [3,6,7,8,9,10]. GHVs have occasionally been detected in both healthy and sick donkeys and mules, including those with respiratory, neurological, or abortion signs [11]. Asinine gammaherpesviruses, such as asinine herpesvirus 2 (also known as *Equid herpesvirus 7*), 4 and 5 (AsHV-2, -4 and -5, respectively) have been identified as individual infection [3,10,12,13,14] or in association with pulmonary fibrosis in donkeys [3,4].

The age of disease onset, its clinical progression, and poor response to medical treatment are recognized as similar features of donkey pulmonary fibrosis and equine multinodular pulmonary fibrosis (EMPF) in horses [15]. EMPF is related to extensive nodular interstitial fibrosis in the adult horse and associated with equine herpesvirus type 5 (EHV-5) infection [4,6,7]. EHV-2 and -5 co-infections are considered cofactors that exacerbate clinical signs [3,4,16]. Although the pathophysiology of EMPF is largely unknown [9,15], a diagnosis can be reached based on changes in thoracic radiographs and ultrasonographic images, histologic examination, and detection of EHV-5 in bronchoalveolar fluid or post-mortem lung tissue [5,7].

The relationship between Epstein-Barr virus (EBV), cytomegalovirus, human herpesviruses (HHV-7 and -8) and pulmonary fibrosis has been described in humans [8] and, similarly, EHV-5 and EMPF have been associated in equids. Equids and rodents affected by GHVs were proposed as a model of several diseases in human medicine including interstitial pulmonary fibrosis [1,6,7,15,17]. Moreover, the donkey may represent a unique experimental model to elucidate the etiopathogenesis of pleuroparenchymal fibroelastosis (PPFE), a human lung disease [17].

The aim of this report was to describe the clinical and histomorphological features of an unusual case of pulmonary fibrosis and first detection of asinine herpesviruses from a donkey (*Equus asinus*) in northern Italy.

## 2. Case report

### 2.1 Case history, clinical findings, and diagnostic examinations

A 23-year-old female domestic donkey, weighing 185kg and with a low body condition score (BCS, 2/9), was admitted with suspected pleuropneumonia at the Internal Medicine Unit (Department of Veterinary Medicinal Sciences, University of Bologna, Italy) in September 2022. The donkey was adopted from a donkey shelter and lived on a farm in Emilia Romagna (Italy) for three years, with three other female donkeys.
Owners provided informed consent for the veterinary treatment and for the donkey data to be used for future publication.
Since 2015, the donkey had a history of asthma and thrombocytopenia (76 $x10^9$plt/L, reference range RR 95-384 platelets/L), but no further diagnostic investigations were made. In August 2022, the donkey presented depression, weight loss, and respiratory distress. Blood tests revealed thrombocytopenia (65 $x10^9$plt/L; RR 95-384 $x10^9$/L) and increased basal ACTH concentration (177 pg/mL; RR 20-50 pg/mL). Therefore, treatment for severe asthma and pituitary pars intermedia dysfunction (PPID) was started by the referring veterinarian.

In September 2022, the donkey was referred because of severe and refractory signs of respiratory distress. On admission, the clinical examination revealed depression, a BCS of 2/9, pale mucous membranes, tachycardia (84 bpm, RR 36-52 bpm), respiratory distress characterized by mixed dyspnea, increased respiratory rate (28 breaths/min, RR 12-28 breaths/min), intercostal retraction and dilated nostrils, associated with mucopurulent nasal discharge, and dry cough. On auscultation of the chest, there was a decrease in bronchovesicular sounds in the cranio-ventral fields, without wheezing and crackling. Rectal palpation was within normal limits. The main abnormalities on hematology and biochemistry analysis were a mild neutrophilic leukocytosis, severe thrombocytopenia (15 $x10^9$ plt/L, RR 100-600$x10^9$plt/L), signs of inflammation with increased serum amyloid A (SAA) concentration (282 µg/dl, RR 1.8–14.5 µg/dl), and hyposideremia (22 µg/dl, RR 55-260 µg/dl) with decreased ferritin saturation (8.6 %, RR 12-70%). Arterial blood gas analysis revealed a mild respiratory acidosis characterized by pH 7.33, severe hypoxemia (PaO2 40mmHg), hypercapnia (PaCO2 61 mmHg), and a mild hypokalemia (3.4 mmol/L mEq/L), hypocalcemia (1.58 mmol/L), and increased base excess (6.8mmol/L). Thoracic radiography revealed a diffuse radiopacity due to pleural effusion. Thoracic ultrasonography (Fig.1) revealed the presence of anechoic pleural fluid (2.5 cm in depth) in the ventral part of the chest, compatible with a mild bilateral pleural effusion. The pleuropulmonary surface was irregular, with multiple hypo/heteroechoic subpleural to irregularly shaped areas consistent with pulmonary consolidations (1-1.5 cm diameter in the left hemithorax; 2.5-3 cm diameter in the right hemithorax). The abdominal ultrasound examination was normal.

Airway endoscopy revealed bronchopulmonary inflammation due to a dense muco-purulent secretion and mucosal edema from the upper airways to the large bronchi. Cytology of a transendoscopic tracheal wash

revealed high-grade neutrophilic inflammation, with a septic nature and a bacterial culture yielded *Streptococcus equi subspecies zooepidemicus* colonies, consistent with a bacterial infection. A bronchoalveolar lavage was not performed due to the severe respiratory distress. A percutaneous lung fine needle aspiration was collected for cytology. The sample was poorly cellular and revealed clusters of epithelial cells with moderate features of nuclear activation, admixed with a fewer number of neutrophils, inducing a suspicion of epithelial neoplasia.

Based on overall findings, the donkey presentation was consistent with a severe bilateral chronic pneumonic disease with a more likely secondary bacterial infection. Differential diagnoses primarily considered were viral or bacterial pleuropneumonia, severe equine asthma, pulmonary fibrosis and a neoplastic syndrome, that was reinforced by the presence of thrombocytopenia.

### 2.2 Treatments, follow-up and case outcome

The donkey underwent oxygen therapy and standard medical treatment with broad spectrum antibiotics while pending susceptibility test, anti-inflammatory, bronchodilators and mucolytic drugs, gastroprotectant, and fluid, electrolytic and nutritional support (Table 1).

**Table 1. Medical treatments administered to 23-year-old donkey presented for respiratory distress upon admission.**

| Treatments (active ingredient) | Dosage, route, time |
|---|---|
| Oxygen therapy | 3 L per minute, intranasal |
| Salbutamol | 500 mcg nebulised, intranasal, q12h |
| Acetylcysteine | 3 ml of 10% solution nebulized, q12h |
| Sodium ampicillin | 20 mg/kg iv q6h |
| Gentamicin sulphate | 6.6 mg/kg iv q24h |
| Lactate Ringer | 2.0 ml/kg/h iv |
| Dextrose 50% | 0.5ml/kg/min iv |
| Flunixin meglumine | 1.1 mg/kg iv q12h |
| Sucralfate | 20 mg/kg q8h po |
| Pergolide | 0.25 mg/day po |

Despite medical treatments the clinical condition worsened. Severe respiratory distress was characterized with further increased respiratory rate (32-36 breaths/min) and abdominal effort. On arterial blood gas analysis there was marked respiratory acidosis: pH 7,242, $PaO_2$ 32.8 mmHg, $PaCO_2$ 75.3 mmHg. In 48 hours of hospitalization the donkey progressed to recumbency and death.

### 2.3 Post-mortem examination and diagnostics

A complete post-mortem examination was performed. Pathological findings included pleural involvement and lungs alterations. There were no pathologically relevant abnormalities affecting the other organs. Pleural effusion and pleuritis were detected. The lung was whitish, collapsed, and firm, with multifocal nodular pinkish areas of 1 to 6 cm (Fig.2.1). On cut section, the lung parenchyma adjacent to the nodules was markedly replaced by abundant fibrosis and the nodular lesions were characterized by residual lung parenchyma spared from fibrosis (Fig.2.2).

Histologically, the pleura was thickened up to 10 times normal with abundant fibrosis and granulation tissue. The lung architecture was markedly replaced by interstitial fibrosis (Fig.2.3). Fibrin, degenerated neutrophils (Fig.2.4), fewer macrophages, and occasional syncytial cells were present in the lumen of residual alveoli and bronchioles. Interspersed with the fibrosis were multifocal residual alveolar structures with type II pneumocyte hyperplasia. Areas of unaffected alveoli were present (Fig.2.5). Overall, the histological features observed were suggestive of a severe chronic fibrosing interstitial pleuropneumonia with type 2 pneumocyte hyperplasia and intralesional syncytial cells, suggestive of a possible herpesviral infection.

On biomolecular investigations, the fibrotic lung tissue tested positive by two pan-herpesvirus polymerase chain reaction (PCR) assays, targeting the partial DNA-directed DNA polymerase (DPOL) and the glycoprotein B (gB) genes, and two sequence of the AsHV-5 and AsHV-2, respectively, were identified from the same tissue sample; real-time PCR assays for EHV-1, -2, -4, and -5 tested negative. The attempts to isolate AsHVs using the cell lines gave negative results.

## 3. Discussion

The identification of asinine gammaherpesviruses in lung tissue associated with interstitial fibrosing pneumonia was herein reported for the first time in a domestic donkey in northern Italy. The diagnostic-therapeutic course of interstitial fibrosis in a donkey is challenging for equine practitioners. The initial respiratory signs of pulmonary fibrosis may be difficult to identify ante-mortem in Donkeys [5, 18] due to their physiological differences such as a reduced pulmonary resistance [5]. Moreover, the reduced cough reflex, the lack of athletic activity and their stoicism probably allow donkeys to cope with a reduced lung function, making it harder to identify [19].

In the presented case, the clinical picture was consistent with the end-stage of a chronic interstitial lung disease, except inspiratory dyspnea was not as evident as it is described in donkeys with pulmonary fibrosis [5,7]. Inspiratory dyspnea might have been masked by other factors than fibrosis, thus altering the respiratory pattern towards a less specific one.

The old age may be considered as an additional risk factor in the reported case, as in human patients with pleuroparenchymal fibroelastosis (PPFE), a rare condition of idiopathic pulmonary fibrosis (IPF) [17].
Concerning collateral findings, there are no reported cases of donkey with pulmonary fibrosis including the arterial blood gas interpretation which revealed respiratory acidosis along with hypoxemia, that progressed to an uncompensated condition. The hypercapnic respiratory failure is reported in the arterial blood gas of human patients with PPFE [17,20].

Another interesting finding in this case was the persistent and severe thrombocytopenia.
To the author's knowledge, there are no other reports of equids affected by pulmonary fibrosis, AsHVs-2 and -5 infection, and thrombocytopenia without other hematological alterations. Thrombocytopenia is commonly associated with clinical conditions including infectious, immune-mediated or neoplastic disorders [21]. In human medicine, a case of a 66-year-old woman with severe thrombocytopenia was reported in association with an active Herpesvirus simplex virus-2 (HSV-2) infection [20]. The mechanisms underlying the HSV-2 as a primary cause of thrombocytopenia is unknown [21]. Some viruses may affect platelet production or destruction, interacting with receptors on the surface or reducing the expression of thrombopoietin receptors [21]. Thrombocytopenia was considered mainly supportive of a paraneoplastic syndrome in this case. A metastatic disease originating from an ovarian tumour in a mare with EMPF was reported [15], but data available concerning paraneoplastic syndrome are limited in donkeys.

In light of the clinical presentation, equine asthma syndrome, bronchopneumonia and pulmonary fibrosis were included in the diagnostic differentials. Similarly, in presence of chronic lower respiratory tract disease in horses, EMPF should be included in the differential diagnosis [15]. To diagnose EMPF in horses, imaging and (transtracheal aspirates and bronchial lavage) biomolecular testing for EHV-5 from airways secretions have been used [16]. Thoracic radiography can be helpful to discern asthma and pulmonary nodular fibrosis [15]. However, in our case a diffuse radiopacity related to pleural effusion hampered the X-ray diagnostic value in recognizing a certain lung pattern. Due to the deteriorating clinical condition, it was not possible to repeat the radiographic exam. On ultrasound examination of the chest, multiple distinct surface nodules of variable size, with comet-tail artefacts can be consistent with EMPF. Furthermore, no pleural effusion has been reported in EMPF [4, 6, 15] in contrast with the present donkey, but it cannot be excluded that it may have been related to the bacterial infection. The ultrasonographic appearance of pulmonary fibrosis in donkeys is noticeably different from EMPF in horses. In donkeys, ultrasound can prompt a suspicion of pulmonary fibrosis if pleural thickening areas are observed but similarly it does not allow a confirmatory diagnosis [5], as in our case. Computed tomography (CT) and magnetic resonance imaging (MRI) are reported

to be useful to diagnose pulmonary fibrosis in human patients [17], however these imaging modalities were not accessible for the donkey.

Features of bronchoalveolar lavage fluid (BALF) cytology in AsHV-4 or -5-infected donkeys are comparable to those in horses with EHV-5-related EMPF, albeit higher macrophages percentages have been observed in donkeys [17]. However, BALF cytology may not allow to discriminate EMPF from other chronic lung diseases (i.e. equine asthma) [15]. Unfortunately, in our case a BAL was planned but not performed due to the severe and deteriorating respiratory condition. The tracheal aspirate was not PCR-tested for GHV due to financial constraints in the face of the clinical condition and the limited therapeutic options. The lack of PCR testing for GHV on tracheal aspirate and/or BALF represents a main limitation of the study. Nevertheless, in horses, it has been showed that the absence of EHV-5 identified in BALF or in lung biopsy samples did not rule out a diagnosis of EMPF [15]. The tracheal fluid was only diagnostic for a *Streptococcus equi subsp. zooepidemicus* infection, that was considered most likely to be secondary to the underlying lung pathology. PPID is not uncommon in older donkeys [22] and could represent an additional risk factor for pulmonary infection [22, 23].

Lymphoma was reported in a horse with concomitant EMPF due to EHV-5 with an unknown causal relationship [24]. The finding of an increased number of epithelial cells arranged in clusters and characterized by nuclear activation has led to misinterpretation and an incorrect diagnosis of neoplasia. The clusters of epithelial cells are due to the hyperplasia of the pneumocytes, found with the histological examination. To avoid misdiagnosis, epithelial hyperplasia should be considered as cytological differential diagnosis when examining donkeys with the present clinical presentation. The necropsy examination of the entire body, together with the histological examination of multiple sections from all the lung lobes, allowed the exclusion of neoplasia.

In horses, donkeys and humans, lung fibrosis has been associated with EHV-5, AsHV-4 and -5, and different GHVs, respectively [15]. Horses are usually infected with EHV early in life and GHV is believed to establish its latency in the lymphatic system [15]. In the present case, the possible contact with EHV infected equine or asymptomatic donkeys could not be excluded due to the adoption from a donkey shelter early in life. Viral factors expressed by viral genes, host factors and co-infections could influence the development of pulmonary fibrosis [15,25]. In horses the EHV-5's causative role in EMPF has been recognized [4, 7, 16]. The clinical significance of AsHV-5 in horses has yet to be defined and other lung diseases such as pyogranulomatous pneumonia have been related to EMPF [18]. The pathogenetic mechanism of pulmonary fibrosis may be due to inflammation, epithelial cell injury, abnormal fibroproliferation and deposition of extracellular matrix components [7,15].

Corticosteroids have been used to treat EMPF and pulmonary fibrosis in horses but owing to their immunosuppressors effect, they could reactivate latent GHVs and indirectly promote viral replication [6, 15]. In this case, corticosteroid treatment was avoided to reduce the risk of an iatrogenic reactivation of latency. Antiviral and antifibrotic are reported to treat pulmonary fibrosis on the early stage of infection in horses with EMPF [6]. The lack of effective therapies is one of the limitations in human medicine, associated with the difficulty of detecting the virus in at-risk patients before the development of fibrosis [2]. Similarly, in the present case, the medical treatments were not effective and the patient was referred when the fibrosis was advanced and it was difficult to identify at an early stage.

Interestingly, in the present case, ultrasonography did not detect a typical nodular pattern of consolidation. Indeed, the macroscopic and histological pattern of fibrosis was diffuse and interstitial, and the nodular lesions were consistent with spared lung parenchyma, instead of the canonical nodular distribution of the fibrosis, hallmark of the equine multinodular pulmonary fibrosis in horses [7]. In fact, in the donkey, compared to the horse, a nodular pattern of fibrosis is not described, but mostly an interstitial pneumonia localized predominantly in the ventral lobes [3]. Instead, the macroscopic features observed in our case are similar to those reported by Miele and colleagues, who interpreted pulmonary fibrosis in 19 donkeys as analogous to human pleuroparenchymal fibroelastosis [17]. However, while pleuroparenchymal fibroelastosis in humans has no specific etiology and is thought to be idiopathic [26], Miele and colleagues identified AsHV-4 and AsHV-5 both in cases with pulmonary fibrosis and in unaffected controls [17]. Conversely, Kleiboeker and colleagues suggested AsHV-4 and AsHV-5 as a cause of interstitial pneumonia in donkeys, being negative for herpesviruses animals affected from pneumonia of different proved etiology [3]. To date, the role of herpesviruses in determining the disease remains object of debate. Eosinophilic intranuclear inclusion bodies were not present in the lung tissue of the present case. According to the literature, herpesviral inclusion bodies are usually not detected in the donkey [10,27], but are generally visible in histological sections of horses with multinodular pulmonary fibrosis [7,28].

In donkeys, AsHV-5 has been associated with fatal respiratory disease and a post-mortem diagnosis of interstitial pneumonia [3]. GHVs (AsHV-2 and AsHV-5) were previously isolated in the Pantesco breed donkey, an Italian breed threatened by extinction, in the Sicily region, Italy [13]. However, GHVs in the donkeys were not considered the sole cause of the respiratory manifestation. The authors supposed that respiratory infection caused reactivation of the herpesvirus [13]. In our case, AsHV-2 and -5 were detected for the first time in a domestic donkey in Emilia Romagna and could be considered in relationship with pulmonary fibrosis, being associated with the canonical histological hallmarks that occur during herpesvirus infection.

## 4.Conclusion

Asinine gammaherpesviruses were detected in association with pulmonary fibrosis for the first time in Emilia Romagna in a domestic donkey and, for the second time, in the asinine population in Italy. The clinical signs and diagnostic findings in the present case add further characterization of the pulmonary fibrosis in this species. The presence of GHVs has been related to pulmonary fibrosis, a chronic and progressive disease, in the present case characterized by acute exacerbation and rapid clinical deterioration. The exact role of GHVs, as a causative or precipitating factors in the pathogenesis of pulmonary fibrosis remains a matter of debate. The present case suggests the importance of considering as a differential and testing donkeys for AsHV-2/5 early in the course of lower respiratory tract diseases.

**Declaration of interest:** None.

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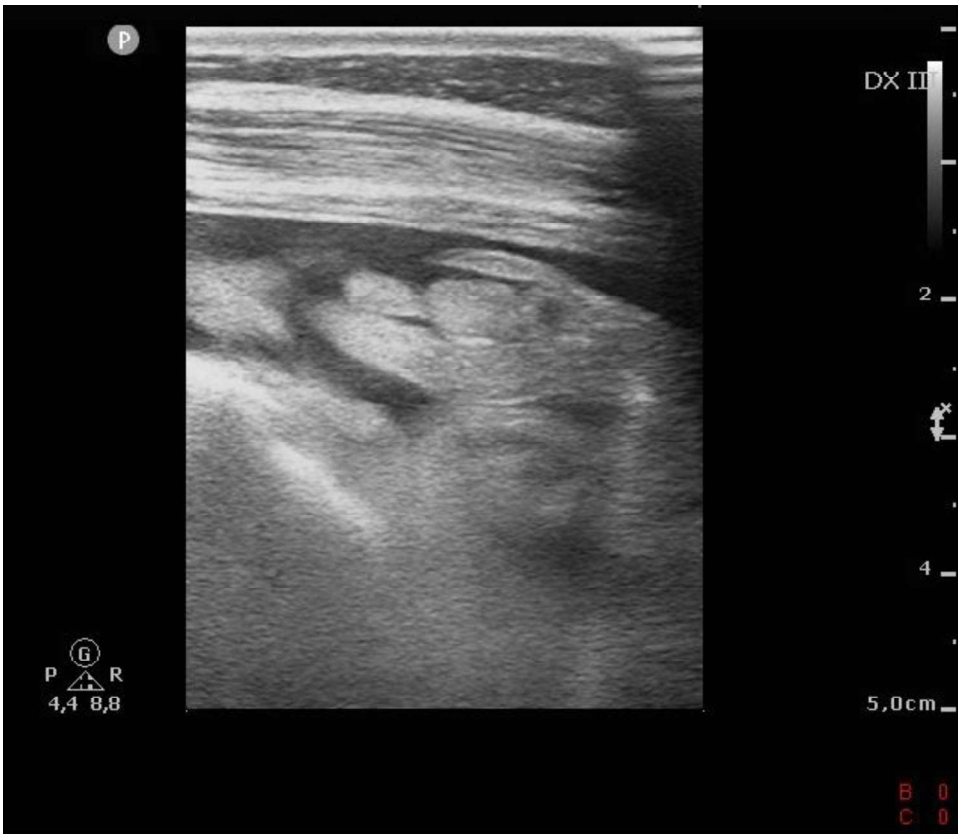


**Figure 1.** Ultrasonographic image of the right thorax, within the third intercostal space, at the first day of hospitalization showing the presence of anechoic pleural fluid and irregular and heterogeneous echogenicity pleuropulmonary surface.

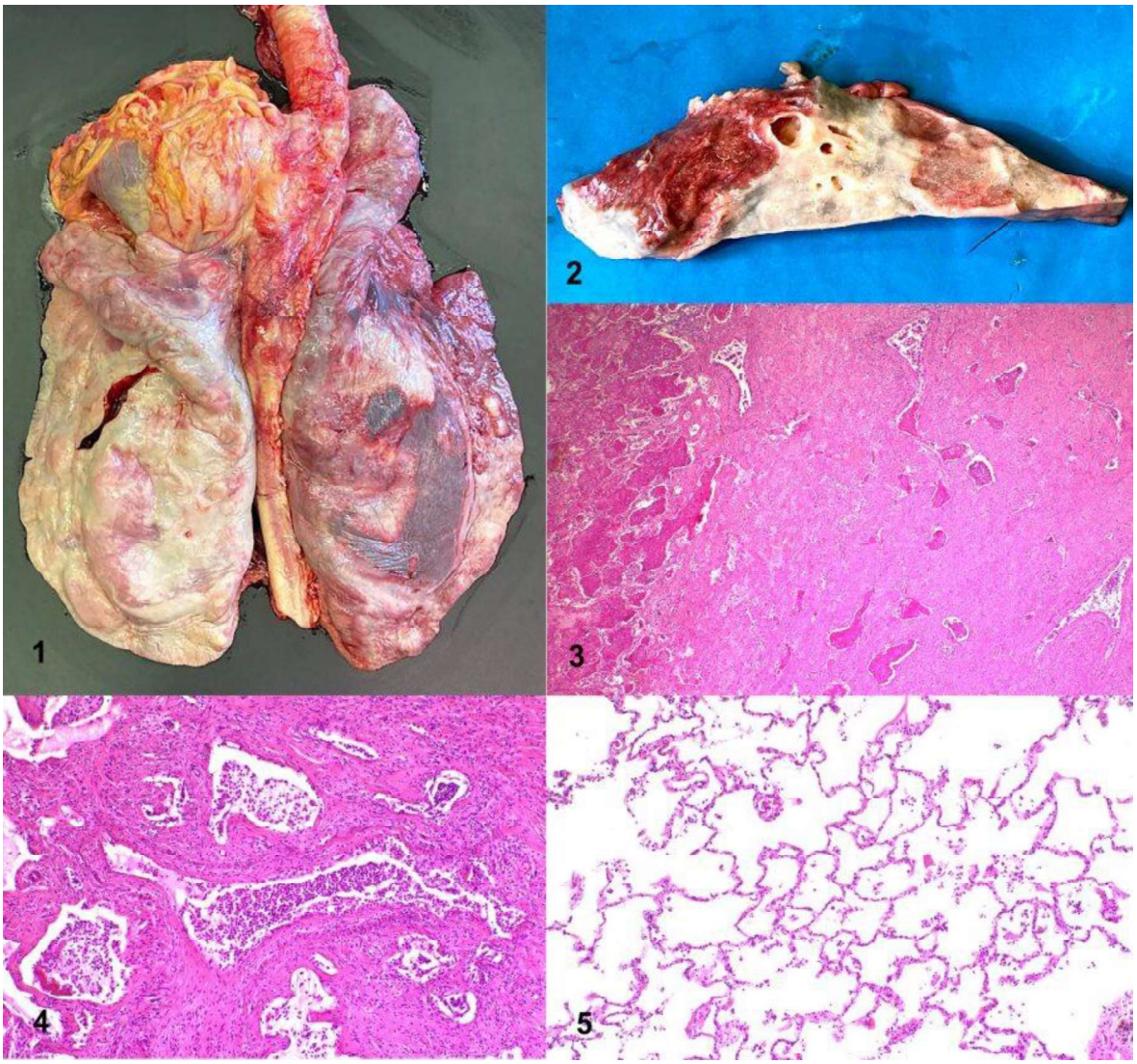


**Figure 2. Post mortem examination of the lungs from a 23-year-old donkey presented for respiratory distress**

**2.1**The lung is whitish and collapsed, with multifocal pinkish nodules.

**2.2** Cut section of the lung: the whitish area is depressed and firm on cut section, suggestive of fibrosis; the reddish area corresponds to the section of a nodule, and it is composed of spared lung parenchyma not affected by fibrosis.

**2.3** The lung architecture is markedly replaced by interstitial fibrosis. The residual alveoli and bronchioles are filled with abundant fibrin and inflammatory cells, 40x, Haematoxylin and Eosin (H-E).

**2.4** Higher magnification of residual airways filled with degenerated neutrophils, 100x, H-E.

**2.5** Alveoli spared from fibrosis, 100x, H-E.

**Supplementary Materials**

**Figure 3** The 23-years-old donkey on admission.