A case series highlighting the role of different gamma-herpesviruses in Equine Multinodular Pulmonary Fibrosis

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Summary

This case series describes three cases of equine multinodular pulmonary fibrosis (EMPF) diagnosed at the Clinic for Equine Internal Medicine at the University of Zurich between 2012 and 2017. Current information on etiology and treatment options are presented. Two horses showed mild signs of chronic lower respiratory tract disease and one horse was presented with acute signs of disease including recurrent fever spikes and tachypnea. Diagnosis was achieved by physical examination, radiographic findings, and PCR testing for equine herpesviruses (EHV) of bronchoalveolar lavage (BAL) fluid or lung tissue obtained by biopsy. All horses were euthanized due to continuing deterioration after attempted treatment. Post mortem histological examination of lung tissue showed severe multifocal diffuse to confluent fibrosis in two cases and in another horse a discrete nodular fibrosis pattern. Panherpes nested PCR revealed the presence of equine herpesvirus 5 (EHV-5) DNA in lung tissue of one horse whereas in two other horses, asinine herpes virus 5 (AHV-5) was detected. EMPF should be considered as a differential diagnosis in horses with acute and chronic respiratory disease, including horses non-responsive to treatment for equine asthma.

Keywords: asinine herpes virus 5; equine herpesvirus 5; horse; interstitial pneumonia; pulmonary fibrosis

Die Rolle von verschiedenen Gammaherpesviren in einer Fallserie von Equiner multinodulärer pulmonalen Fibrose

Diese Fallserie beschreibt drei Fälle von Equiner multinodulärer pulmonaler Fibrose (EMPF), die zwischen 2012 und 2017 an der Klinik für Innere Medizin für Pferde der Universität Zürich diagnostiziert wurden. Der aktuelle Wissensstand zur Ätiologie und Behandlungsmöglichkeiten werden vorgestellt. Zwei Pferde zeigten leichte Anzeichen einer chronischen Erkrankung der unteren Atemwege. Ein Pferd hatte Anzeichen einer akuten Erkrankung mit Fieberschüben und Tachypnoe. Die Diagnose konnte durch die klinische Untersuchung, radiologische Befunde und die Untersuchung der bronchoalveolären Lavage (BAL) oder Lungenbiopsie auf Equine Herpesviren (EHV) mittels Polymerase Kettenreaktion (PCR) bestätigt werden. Aufgrund der klinischen Verschlechterung mussten alle Pferde euthanasiert werden. Pathohistologische Untersuchungen des Lungengewebes zeigte eine schwere multifokale diffuse bis konfluente Fibrose in zwei Fällen und ein diskretes Muster von knotiger Fibrose in einem Fall. Mittels Panherpes PCR (Nested PCR) konnte bei einem Pferd DNA von Equinem Herpesvirus 5 (EHV-5) und bei zwei Pferden Asinin-Herpesvirus 5 (AHV-5) in der Lunge nachgewiesen werden. Bei Pferden mit akuten und chronischen Atemwegserkrankungen, oder Pferden, die nicht auf die Behandlung von Asthma ansprechen, sollte EMPF als Differenzialdiagnose in Betracht gezogen werden.

Schlüsselwörter: Asinin-Herpesvirus 5; Equines Herpesvirus 5; Pferd; interstitielle Pneumonie; Lungenfibrose

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Introduction

Equine multinodular pulmonary fibrosis (EMPF) is a rare interstitial lung disease affecting horses at all ages.[1-16] The etiology is not fully elucidated, but gamma herpes viruses likely play a major role. Gamma herpesviruses have been shown to induce pulmonary fibrosis in experimental murine γ-herpesvirus 68 (MHV68) models (officially murid herpesvirus type 4 (MuHV-4)), as well as in humans with idiopathic pulmonary fibrosis (Epstein-Barr Virus (EBV) infection).[17-19] Experimental infection with equine herpes virus 5 (EHV-5) has also been shown to induce pulmonary fibrosis in horses in vitro and in vivo.[20] The first published case series from 2007 described coinfection with EHV-2 in approximately 30% of the cases.[12] Co-infection with EHV-2 has since been described in approximately 20% of cases (Table 1). EHV-2 and EHV-5 belong to the family of gamma herpesviruses, which also include equine herpesvirus 7 (EHV-7, also referred to as asinine herpesvirus 2 (AHV-2)) and the closely related asinine herpes viruses 4 (AHV-4), asinine herpes viruses 5 (AHV-5) and asinine herpes viruses 6 (AHV-6).[21,22] AHV-2 has also been detected by PCR in lung tissue of EMPF affected horses,[3,23] and AHV-5 has been associated with a case of pyogranulomatous pneumonia in a horse.[23] Clinical relevance and role in pathogenesis of these viruses is still unclear.[20,24] Synergism of different gamma herpesviruses in pathogenesis could potentially occur.[19,25] Clinical signs of EMPF depend on the stage of disease and include tachypnoea, increased respiratory effort, tachycardia, weight loss/poor overall condition, intermittent fever and cough.[12,26] Inflammatory changes and hypoxemia can be seen on hematology and biochemistry.[26,27] Diagnosis is based on distinct changes on thoracic radiographs and ultrasonographic images histologic examination, as well as detection of EHV-5 in BAL fluid (BALF) or lung tissue.[12,26] There is little information on treatment of EMPF. Corticosteroid, acyclovir or valacyclovir and doxycycline therapy for several weeks are described but the long-term prognosis of EMPF remains poor.[2,26,27] A recent case series described short-term survival as 57% in 14 cases, with only 14% surviving longer than 6 months after diagnosis.[2] This case series provides further information on the association of different gamma herpesviruses with EMPF and highlights the different clinical features of EMPF. EMPF has to be kept in mind as a differential diagnosis for horses with acute or chronic lower respiratory tract disease including equine asthma syndrome or bronchopneumonia.

Cases

Case 1

A 23-year-old Irish Warmblood mare was presented in June 2017 due to persistent anorexia, weight loss and exercise intolerance of three months duration. The re-

Table 1: Summary of published equine multinodular pulmonary fibrosis (EMPF) case reports with PCR diagnosed coinfection of equine herpesvirus (EHV)-5 and EHV-2 or asinine herpesvirus (AHV)-5 between 2007 – 2019.

		Positive PCR lung tissue		
	Number of cases	EHV-5	EHV-2	AHV-5
Williams et al. 2007[12]	24 (23 control)	24 (0 control)	8 (1 control)	
Hart et al. 2008[6]	1	1	0	
Wong et al. 2008[26]	5	5	0	
Belgrave et al. 2009[47]	5	5	0	
Poth et al. 2009[9]	5	4	3	
Niedermaier et al. 2010[8]	2	2	1	
Verryken et al. 2010[48]	1	1	0	
Lehmbecker et al. 2011[7]	1	1	1*	
Soare et al. 2011[13]	2	2	0	
Tomlinson et al. 2011[15]	1	0	0	
Back et al. 2012[3]	1	1	0	1
Gomez de Witte et al. 2012[23]	1	0	0	1
Schwarz et al. 2012[10]	1	1	0	
Schwarz et al. 2013[1]	5	5	0	
Schwarz et al. 2013[11]	1	1	0	
Spelta et al. 2013[14]	3	3	0	
Dunowska et al. 2014[5]	1	1	0	
Easton-Johnes et al. 2019[2]	14	12	0	
Total	**74**	**69**	**13 (18.8%)**	**2**

* only in small amounts on Real-Time PCR detected

ferring veterinarian palpated an abdominal mass on rectal examination two days prior to presentation, which was suspected to be a focal impaction. A dental examination was without abnormal findings and the mare was dewormed with ivermectin three months prior to presentation (dose unknown, PO). As the abdominal mass persisted despite laxative therapy with mineral oil, the mare was referred for further diagnostics and treatment. No behavioral changes were reported and the mare was bright, alert and responsive. The mare had a thin body condition score (3/9), normal rectal temperature (37.6 °C) and heart rate (36 beats/min). Respiratory rate was slightly elevated (18 breaths/min). There was no increased inspiratory effort and the horse showed a normal costoabdominal respiratory pattern. On lung auscultation, mild to moderate bilateral increased bronchovesicular sounds were heard, crackles and wheezes were absent. Mild bilateral serous nasal discharge was seen but cough was absent. The remainder of the physical examination was within normal limits. On rectal examination the abdominal mass was diagnosed as an enlarged right ovary (12 × 10 × 10cm). On rectal ultrasonography it measured 10 cm in diameter with a honeycomb structure. The performed hematology and biochemistry analysis showed clinically significant abnormalities including hyperfibrinogenemia, hyperglobulinemia and an elevated SAA (Table 2). A working diagnosis of a granulosa thecal cell tumor or ovarian hematoma and chronic lower respiratory tract disease was made. Diagnostics of the ovarian mass were pursued first to rule out neoplastic disease. Thoracic radiographs and abdominal paracentesis were performed to evaluate the presence of metastatic abdominal or lung disease. There were no neoplastic cells evident on cytologic examination of the abdominal fluid. A generalized severe interstitial lung pattern with both a structured nodular and an unstructured pattern, was diagnosed (Fig. 1A).

EMPF or metastatic disease originating from the suspected ovarian tumor were considered as differential diagnosis for the radiographic findings. Additional results became available several days later and included a normal Anti-Müller hormone ruling out granulosa cell tumor (0.23 ng/mL Ref range <4 ng/mL)[28] and normal thymidine-kinase concentrations (tumor marker), making a tumor unlikely (1.4 U/L Ref range < 2.7 U/L).[29] Cytologic examination of a fine needle aspirate of the right ovary mass per rectum was consistent with an ovarian hematoma. Neoplastic disease of the ovaries was ruled out and further diagnostics for the respiratory disease were pursued. Ultrasonographically, the pulmonary surface was moderately irregular producing comet tail artefacts, and multiple subpleural nodules of approximately 2 to 5 cm in diameter were found (Fig. 1B). On arterial blood gas analysis, hypoxemia was present (Table 2). Performing airway endoscopy, moderate amounts of white, viscous mucus (amount: 2.5/5; color: 3/5; apparent viscosity 3.5/5)[30] and a thickened carina were visualized. Cytological evaluation of a tracheal aspirate (TA) was consistent with severe chronic neutrophilic lower airway inflammation (Table 2). Bronchoalveolar lavage (BAL) confirmed the diagnosis of mild neutrophilic lower airway inflammation with activated macrophages (Table 2). A panherpes nested PCR of BAL secretion with subsequent sequencing of the PCR product revealed an inconclusive result due to superimposed sequences. Cloning of the PCR product showed the presence of EHV-2 as well as AHV-5 (Fig. 2).

Cytological examination of a fine needle aspirate of the lung lesions showed presence of mild mixed inflammation (erythrocytes, neutrophils and macrophages as well as a few mast cells, eosinophil granulocytes, mesothelial cells and high prismatic ciliated epithelial cells were seen, no bacteria or and no neoplastic cells were found). In the examination of the lung biopsy few bronchi, alveolar tissue and collagen connective tissue were seen. The amount of macrophages in the alveoli was increased and they contained several haemosiderophages. Neutrophils, lymphocytes and plasma cells were present in smaller numbers. A mild suppurative and histiocytic pneumonia was diagnosed. A panherpes nested PCR performed on lung tissue samples was positive for EHV-5 (Fig. 2). The diagnosis of EMPF was made. Antiviral therapy was started with valacyclovir (Valaciclovir-Mepha, Mepha Pharma AG Basel, Switzerland, 30 mg/kg, TID, PO for 48 hours, followed by 20 mg/kg TID, PO for 28 days). A tapering course of dexamethasone was added (Dexadreson ad us. vet., MSD Animal Health GmbH Luzern, Switzerland, 0.1 mg/kg SID, PO for 10 days, 0.05 mg/kg SID, PO for 10 days, 0.05 mg/kg q48 h, PO). Penicillin (Penicillin Natrium Streuli ad us. vet., Streuli Pharma AG, Uznach, Switzerland, 30'000 IU/kg QID, IV), gentamicin (Genta-100 ad us. vet., CP-Pharma Handelsges. mbH, Burgdorf, Germany, 9 mg/kg, SID, IV) and flunixin (Flunixine ad us. vet., Biokema SA, Crissier, Switzerland, 1.1 mg/kg SID, IV) were administered for four days and changed to doxycycline (Doxylin 100% ad us. vet., Raamsdonksveer, The Netherlands, 10 mg/kg, BID, PO for 30 days) for prevention of complications from the ovarian hematoma. On recheck examination after one month of antiviral treatment, the mare was lethargic, showed tachypnoea (24 breaths/min) and moderate abdominal effort during respiration. On lung auscultation severe increased bronchovesicular sounds, wheezes and friction rubs were heard on both sides of the lungs. Hypoxemia worsened (from pO_2 72 mmHg to 63.3 mmHg) and fibrinogen and SAA further increased (from 405.6mg/L to 2198.2mg/L). Thoracic radiographs and ultrasound were consistent with progressive EMPF pathology. Based on the progressive deterioration and poor prognosis, the

Table 2: Laboratory results of the three equine multinodular pulmonary fibrosis (EMPF) cases.

	Case 1	Case 2	Case 3	Reference interval
Hematology				
PCV (%)	32	28	39	30-42
Hemoglobin (g/dL)	10.9	10	13.6	10.8-14.9
Erythrocytes (10^3/mL)	6.2	5.89	7.96	6.2-9.0
MCH (pg)	16	17	17	15-18
MCHC (g/dL)	34	36	35	35-37
MCV (fl)	48	47	48	41-50
Leukocytes (10^6/mL)	5.6	12.6	20.5	4.7-8.2
Thrombocytes (10^3/mL)	258	188	220	119-250
Neutrophils (10^3/mL)	4.19	5.71	15.28	3.02-5.78
Segmented neutrophils (10^3/mL)	No data	No data	15.28	3.02-5.78
Band neutrophils (10^3/mL)	No data	No data	0	0.00-0.08
Basophiles (10^3/mL)	0.01	0.01	0.21	0.00-0.07
Monocytes (10^3/mL)	0.16	0.3	0.1	0.00-0.18
Eosinophils (10^3/mL)	0.45	0.02	No data	0-0.22
Anisocytosis	few	No data	few	few
Lymphocytes (10^3/mL)	0.75	0.98	4.92	1.020-3.47
Chemistry				
Fibrinogen (g/L)	7.0**	6	4	1-5 (1.2-2.85**)
Serum amyloid A (mg/L)	405.6	1552.4	No data	0.5-1.2
Total bilirubin (mcmol/L)	30.8	55*	12.8	19-39 (17.4-35.2*)
Glucose (mmol/L)	5.5	4.9	4.7	4.5-5.9
Urea (mmol/L)	4.7	4.6	5.2	3.5-7.0
Creatinine (mcmol/L)	91	102	111	82-147
Protein Biuret (g/L)	60	71	67	57-70
Albumin (g/L)	26	23	26	25-34
Alkaline phosphatase (U/L)	223	289	271	81-183
ASAT (GOT) (U/L)	190	146	227	229-393
GGT (U/L)	13	13	21	6-31
GLDH (U/L)	5.1	3.9	1.1	0.5-2.2
SDH (U/L)	3.6	6.5	3.2	0.1-7.6
CK (U/L)	135	92	502	112-305
LDH (U/L)	702	340	667	369-822
Sodium (mmol/L)	139	138	144	139-147
Potassium (mmol/L)	3.8	3.4	4.3	2.3-4.6
Chloride (mmol/L)	99	97	104	98-106
Calcium (mmol/L)	3.00	2.67	2.81	2.9-3.3
Magnesium (mmol/L)	0.69	0.61	0.68	0.6-0.8
Phosphate (mmol/L)	0.98	1.01	0.87	0.7-1.3
Arterial blood gas				
pH	7.45	7.47	7.44	7.35-7.47
pCO2 (mmHg)	38.2	44	44	36-46
HCO3 (mmol/L)	26.7	29	27.9	22-29
Anion Gap (mmol/L)	13.9	11.8	19.9	9-16#
tCO2 (mmol/L)		30.4	29.3	27-33#
BE (mmol/L)	2.5	5.0	3.7	1.1-7.1#
pO2 (mmHg)	72	78	87	80-100
tHb (g/dL)		12.8	11.9	10-18
sO2 (%)		95	97	93-100

	Case 1	Case 2	Case 3	Reference interval
Tracheal aspirate				
TA neutrophils (%)	++++	No data	+++	((+)) – (+)/32±8.9##
TA macrophages (%)	(+)		(+)	(+)/24±4.0##
TA eosinophils (%)	(+)		(-)	((+))/<1.0##
TA tracheobronchial epithelium cells (%)	((+))		((+))	((+)) – (+)34±6.6##
Broncho alveolar lavage				
BAL cell count (cells/mL)	250	650	688	184±22
BAL neutrophil count (%)	11.0	39	37.3	4.6±1
BAL macrophages (%)	36.5	41	25.4	57.6±4
BAL basophils (%)	0	0	1.4	3.85±0.59
BAL eosinophils (%)	0	0	0	0.29±0.59
BAL lymphocytes (%)	51.5	18.5	35.9	36.0±3
BAL mast cells (%)	1.0%	1.5	0	1.2±0.3#
Serology (Competitive ELISA, Testkit VMRD Inc.)				
B. caballi		5% (-)		≤40% negative###
≥40% positive				
T. equi		4% (-)		
Multiplex PCR				
B. caballi		(-)		
T. equi		(-)		
Blood smear				
B. caballi and T. equi		(-)		

(-) not found, ((+)): rare, (+): few, +: moderate, ++: moderate-lots, +++: lots, ++++: in huge numbers; BAL: bronchoalveolar lavage. BAL cell counts are based on 200 cells; * measured stall side with a point of care (POC) analyzer, in the reference column * indicates the reference range of the POC analyzer; ** New reference range for the in-house laboratory valid after February 8, 2017; # Animal Health Diagnostic Center of Cornell University; ## Derksen *et al* (1989)[49]; ### Competitive ELISA (Testkit VMRD Inc.), results in % of inhibition.

mare was euthanized. Relevant macroscopic findings on post mortem examination was the presence of multifocal to coalescing different sized (up to 5 cm in diameter) whitish, firm nodules in all lung lobes (Fig. 3). Histology findings fulfilled all criteria for a severe multinodular pulmonary fibrosis were seen, such as severe fibrosis of the parenchyma, large amounts of neutrophils and macrophages in the alveolar spaces, pneumocyte type II hyperplasia and some syncytial cells. No intranuclear inclusions could be seen. Panherpes nested PCR of lung

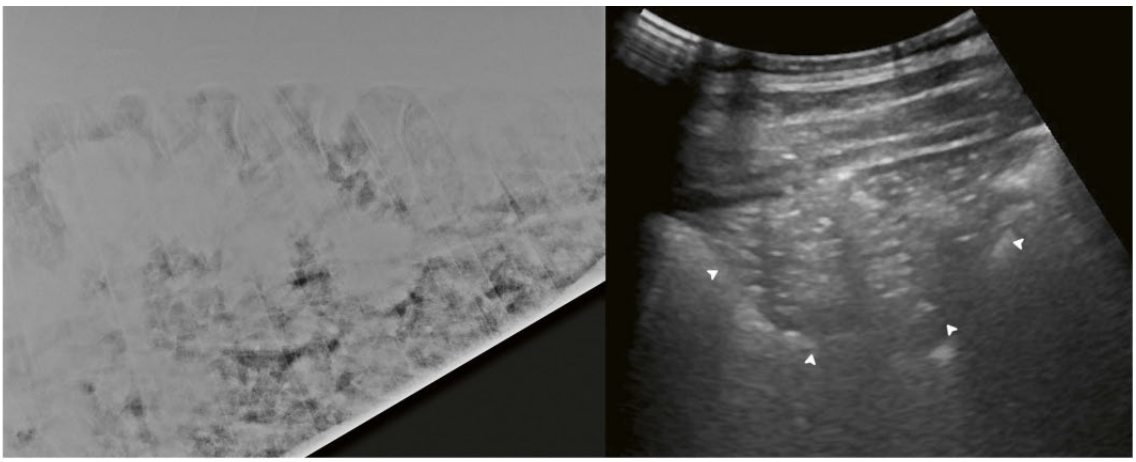

Figure 1A and B: Lateral radiograph of the caudodorsal lung field (A) of 23-year-old Irish Warmblood (case 1) demonstrates a generalized severe unstructured and structured (nodular) interstitial lung pattern. Ultrasound images of the lung (B) in the same horse show a heterogeneous subpleural pulmonary nodule (white arrowheads). During real-time imaging, the nodule was moving synchronously with respiration.

tissue was positive for EHV-2 (Fig. 2). On the cranial pole of right ovary, a blood-filled cavity of 6 × 4 × 4 cm was seen and diagnosed macroscopically and histologically as a hematoma of the right ovary.

Case 2

A 15-year-old Oldenburg gelding was presented in July 2013 with a two-week history of recurrent fever (up to 40 °C), tachypnoea, exercise intolerance and weight loss despite a good appetite. The referring veterinarian treated the horse with broad-spectrum antibiotics (trimethoprim-sulphonamide, dose unknown) and an unknown NSAID (dose unknown) with suspicion of viral or bacterial infection without improvement. At presentation the gelding had a thin body condition score (4/9), an elevated rectal temperature (38.6 °C), tachypnoea (52 breaths/min) and a normal heart rate (40 beats/min). Mild abdominal effort and nostril flaring was present during respiration, but no dyspnea or cough was evident. On lung auscultation increased in- and expiratory bronchovesicular sounds on both sides were heard, crackles or wheezes were absent. Increased sounds on tracheal auscultation were present. Bilateral serous nasal discharge and mild depigmentation of both nostrils were noticed. The remainder of the physical examination was within normal limits. Based on history and physical examination, a diagnosis of lower respiratory tract disease was made, and further diagnostics performed. On arterial blood gas analysis, hypoxemia was present. Hematology and biochemistry analysis showed leukocytosis, hyperfibrinogenemia, and elevated SAA (Table 2). Upper and lower airway endoscopy were without any abnormal findings. Cytology of BAL fluid showed a neutrophilic inflammation with evidence of moderate pulmonary hemorrhage and mildly increased amounts of mucus. No bacteria, fungus or neoplastic cells were present (Table 2). On thoracic radiographs, a generalized severe bronchointerstitial pattern with a focal ill-defined alveolar pattern dorsal to the caudal vena cava and blurred to invisible pulmonary vessels and caudal vena cava were seen. Ultrasonographically, the pulmonary surface was mildly to moderately irregular producing comet tail artefacts, and a small amount of anechoic free fluid was found. Due to fever of unknown origin, treatment was started with penicillin (30'000 IU/kg QID, IV), gentamicin (9 mg/kg, SID, IV), metronidazole (pharmaceutical compounding, Apotheke Tierspital Zurich, Switzerland, 25 mg/kg BID, PO), flunixin (2.2 mg/kg, BID, IV). Over the next five days the gelding did not improve clinically, and further diagnostics were performed. The horse tested negative for blood parasites (Babesia caballi, Theileria equi) on PCR and serology (Table 2). A lung biopsy was performed and showed mild to moderate interstitial lung fibrosis, accompanied by purulent, histiocytic pneumonia of unknown origin. No neoplastic cells, bacteria or fungi were present. A panherpes nested PCR of biopsy material revealed the presence of AHV-5 (Fig. 2). A diagnosis of EMPF was made, and a guarded prognosis given. Antiviral therapy was started with valacyclovir (27 mg/kg, TID, PO for 48 hours, followed by 18 mg/kg BID, PO). Additionally, the gelding was inhaled with salbutamol (Ventolin, GlaxoSmithKline AG, Münchenbuchsee, Switzerland, 10 puffs TID) and fluticasone (Axotide, GlaxoSmithKline AG, Münchenbuchsee, Switzerland, 10 puffs TID) to reduce airway inflammation and provide relieve from dyspnea. Dexamethasone was added (0.05 mg/kg SID, PO). Antibiotics were continued to prevent secondary bacterial infection and changed to exclusively intravenous administration with penicillin (30'000 IU/kg QID, IV) and marbofloxacin (Forcyl ad us. vet., Vetoquinol AG, Bern, Switzerland, 2 mg/kg, BID, IV). As the horse remained febrile, flunixin (1.1 mg/kg SID, IV) was continued. During the next 15 days fever spikes of up to 40 °C occurred. Coughing, severe tachypnoea, dyspnea and anorexia developed. Radiographic and ultrasonographic findings were stationary to mildly worsened. Due to the progressive deterioration and poor prognosis the gelding was euthanized three weeks after the initial presentation. Relevant macroscopic findings on post mortem examination was a severe increased consistency of all lung lobes with bad retraction of the lung and a nodular pattern. Histologically a severe multifocal to coalescing fibrosing, histiocytic and suppurative bronchointerstitial pneumonia with rare viral intranuclear inclusion bodies was present. To quantify the collagen fibers in the lung, a Van Gieson special stain was applied, and an increase of collagen fibers was vis-

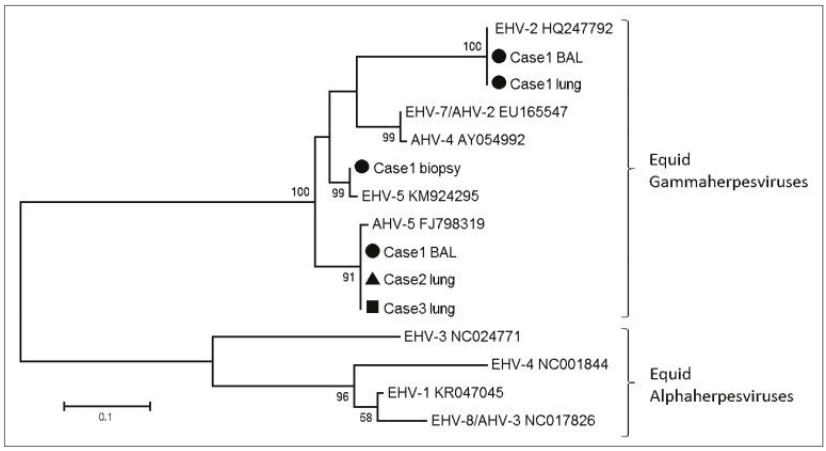

Figure 2: Phylogenetic tree of equid herpesviruses including sequences from the three equine multinodular pulmonary fibrosis (EMPF) cases (black symbols). References were downloaded from NCBI Genbank, where available. The maximum likelihood tree is based on a MUSCLE alignment of 158-170nt long fragments (panherpes nested PCR product) of the polymerase coding region and was tested with 1000 bootstraps. For several equid herpesviruses, such as EHV-6/AHV-1, EHV-9, AHV-6 no references were available that covered the whole fragment length. BAL: Isolated from bronchoalveolar lavage fluid (BALF); lung: isolated from lung tissue obtained during post mortem examination; biopsy: isolated from lung tissue obtained by lung biopsy.

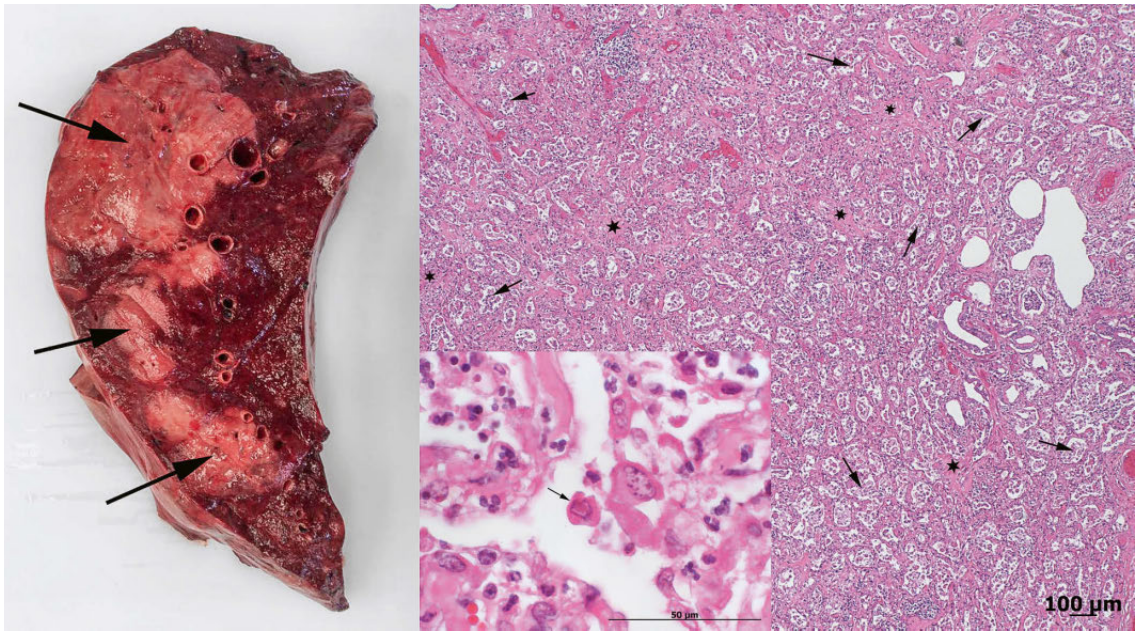

Figure 3A and B: Gross post mortem (A) and histologic lesions (B) of a 23-year-old Irish Warmblood (case 1) with equine multinodular pulmonary fibrosis (EMPF). Note the large discrete nodules of fibrosis with sharp borders (arrows) adjacent to grossly normal lung tissue (A). Note the thickened parenchyma due to severe interstitial fibrosis (asterisks) and less affected lung areas where only few aerated alveolar spaces (arrows) are still visible (B, magnification 4x), Hematoxylin-Eosin (HE) staining. Inset: Arrow: Intranuclear inclusion body (D, magnification 40x), HE staining.

ualized. The panherpes nested PCR of lung tissue confirmed the presence of AHV-5 (Fig. 2).

Case 3

A 15-year-old, Belgium Warmblood mare was presented in November 2012 due to progressive coughing and nasal discharge of two years duration. The referring veterinarian diagnosed the horse with severe equine asthma six months prior to presentation. Treatment consisted of several courses of acetylcysteine (ACC) with or without clenbuterol (both doses unknown, PO) and two courses of prednisolone (unknown dose and duration, PO). Management changes consisted of feed change to silage and grass, bedding change from straw to wood shavings. Show jumping was discontinued and the mare used for pleasure riding only. The clinical signs were unresponsive to treatment and the mare was referred for further diagnostic procedures and therapy. There was no fever reported. On admission, the mare had a normal body condition score (5/9), rectal temperature (37.7 °C), heart rate (40 beats/min) and respiratory rate (16 breaths/min). Mild abdominal effort was present during respiration, but no dyspnea was evident. During a rebreathing test, increased bronchovesicular sounds were auscultated but crackles and wheezes were absent. Rattles were present on tracheal auscultation. Bilateral serous nasal discharge and mild depigmentation of both nostrils were noticed. The remainder of the physical examination was within normal limits. Based on history and physical examination, a presumptive diagnosis of chronic lower respiratory tract disease was made, and further diagnostics were performed. Hypoxemia on arterial blood gas analysis (Table 2) and prolonged recovery time after lunging were indicative of ventilation/perfusion mismatch. On hematology and biochemistry analysis, significant abnormalities included leukocytosis with left shift and lymphocytosis (Table 2). On airway endoscopy moderate amounts of white, viscous mucus and a thickened carina were visualized (amount: 2/5; color: 3/5; apparent viscosity 3/5).[30] Cytological evaluation of a tracheal aspirate (TA) and BAL (Table 2) were consistent with equine asthma (Table 2). A diagnosis of severe equine asthma was made, and treatment started with prednisolone (Prednisolon Vétoquinol ad us. vet., Vetoquinol AG, Bern, Switzerland, 2 mg/kg, 1.5 mg/kg, 1 mg/kg SID, PO, for 10 days each) and ACC (Acetylcystein ad us. vet., Dr. G Bichsel AG, Unterseen, Switzerland, 5 mg/kg BID, PO) and further management changes to ensure a dust and hay free environment were recommended. At recheck examination six weeks later, the clinical signs did not improve and laboratory and endoscopy findings as well as cytological evaluation of TA and BAL were unchanged. On thoracic radiographs, a generalized severe interstitial to miliary lung pattern with peribronchial cuffing and blurring of the pulmonary vessels and the caudal vena cava was seen. Ultrasonographically, the pulmonary surface was moderately irregular producing comet tail artefacts, and multiple small subpleural nodules (2-3mm in diameter) were found. Imaging findings were consistent with EMPF,

granulomatous pneumonia or neoplasia. A sample of BAL fluid was submitted for panherpes nested PCR which was negative. A lung biopsy was performed and showed multifocal interstitial lung fibrosis accompanied by a chronic non-purulent pneumonia. No neoplastic cells were present. The diagnosis of EMPF was made and a guarded prognosis given. Palliative treatment was started with clenbuterol (Ventipulmin, Boehringer Ingelheim GmbH, Basel, Switzerland, 0.8 mcgr/kg BID, PO) and dembrexine (Sputolysin, ad us. vet., Boehringer Ingelheim GmbH, Basel, Switzerland, 0.3 mg/kg BID, PO) and a tapering course of prednisolone (2 mg/kg, 1.5 mg/kg, 1 mg/kg SID, PO for 10 days each). As the mare developed sweating and discomfort which were attributed to side effects from clenbuterol, therapy with clenbuterol was stopped and dembrexine was changed to ACC (5 mg/kg BID, PO) due to convenience. Over the following next months, clinical and laboratory findings were unchanged on recheck examination. Therapy was changed to inhalation therapy (fluticasone 2 mcgr/kg BID) and a repeated course of tapering oral prednisolone was attempted. Initially the owner reported improvement of clinical signs, however the mare worsened when corticosteroid therapy was discontinued. On recheck examination eight months after the initial presentation, clinical signs and hypoxemia had worsened (pO_2: 75 mmHg) and more pathologic lesions were seen on thoracic ultrasound. Based on the deteriorating condition a poor prognosis was given and no further treatment attempted. The mare deteriorated further at home, respiratory signs worsened she became anorexic and unwilling to move. Based on the progressive deterioration, the mare was euthanized nine months after the initial presentation. Relevant macroscopic findings on post-mortem examination were an increased consistency of the lung parenchyma and nodular changes mostly in both diaphragmatic lung lobes. Histological examination revealed a severe multifocal to diffuse chronic granulomatous pneumonia and multifocal interstitial fibrosis, as well as a diffuse fibrosis of the pleura as confirmed by the Van Gieson special stain for collagen fibers. Panherpes nested PCR was performed from lung tissue and showed the presence of AHV-5 (Fig. 2).

Discussion

This case series provides further information about the association of different gamma herpesviruses with EMPF and highlights the different clinical features of EMPF. EMPF has to be kept in mind as a differential diagnosis for horses with acute or chronic lower respiratory tract disease including equine asthma syndrome or bronchopneumonia. Antiviral treatment with valacyclovir in two of three horses in conjunction with supportive therapy did not result in improvement. Remarkable in this case series was the detection of AHV-5 in absence of EHV-5 in two of the cases. Coinfections cannot be excluded completely as the panherpes nested PCR detects only the dominant virus.

Gammaherpesvirinae, which include EHV-2, EHV-5, EHV-7 (also called AHV-2) and the closely related asinine herpes viruses AHV-4, AHV-5 and AHV-6[21,22] may have a role in pulmonary fibrosis as evidenced by experimental murine γ-herpesvirus 68 (MHV68) models, as well as humans with idiopathic pulmonary fibrosis correlated with Epstein-Barr Virus (EBV) infection.[17,18,31] AHV-4, AHV-5 and AHV-6 have been detected in donkeys with interstitial pneumonia, with lesions similar to EMPF.[22,32] Detection of AHV-5 in horses is rarely described. AHV-5 was detected in horses with respiratory disorders or "poor performance syndrome".[24] Concurrent detection of EHV-5 and AHV-5 in an EMPF case has been described in a case report.[3] AHV-5 has also been detected in lung tissue of a horse with pyogranulomatous pneumonia, a pathology differing from described EMPF cases in horses and interstitial pneumonia in donkeys with AHV-5 infection.[23]

Detection technique and virus load may be responsible for the inconsistent results from BALF, lung biopsy and necropsy in two of the cases. A similar finding was reported in a recent case series, where one horse tested negative for EHV-5 on qPCR of lung tissue ante-mortem but proved to be positive for EHV-5 post-mortem.[2] Two other horses were negative on testing of lung tissue for EHV-5 despite characteristic histopathological lesions[2]. Similarly one horse with hypertrophic osteopathy secondary to nodular pulmonary fibrosis also tested negative for EHV-5 on post mortem lung examination.[15] In our case series, EHV-5 was detected only in one case in lung tissue ante- mortem. The other two cases were tested positive for AHV-5 in lung tissue post-mortem.

Panherpes Nested PCR is a sensitive technique to identify a broad range of herpesviruses. This method targets a highly conserved region of the herpesviral DNA polymerase gene. Using degenerate consensus primers, PCR is able to not only detect known herpes viruses, but also novel herpesviruses without information on DNA sequence.[33] Sequencing of PCR products is impaired if the viral load is very low. Incomplete reads and incorrect incorporation of bases by Taq DNA polymerase may occur.[33] Co-infections may often be overlooked as primarily the sequence of the dominant virus will be identified in a panherpesvirus PCR. Superimposed sequences and double peaks in electropherograms are indications for co-infections. However, only subsequent cloning allows separation of the sequences and hence determination of the individual viruses. In case 1, three different equid gamma herpesviruses were detected. If this

reflects different virus localization/excretion patterns (BAL versus lung tissue) or serial circulation of the viruses cannot be conclusively answered. Induction of fibrosis occurs during latency of the virus in the lungs. In this phase, low virus DNA copy numbers beyond detection limits of the assay may be present.[34] Viral load in BALF may have been reduced in some of our cases due to prior medication, which has also been shown in another study.[26] On the other hand, administration of steroids may reactivate latently present gamma herpesviruses that are not causally involved in the generation of EMPF, such as EHV-2. If and how the reactivation of these viruses may influence the clinical outcome is unclear.

Gamma herpesviruses are suspected to be involved in pulmonary fibrosis in humans, rodents, and domestic animals (dog, cat, horse).[35,36] There are similarities between fibrotic disease in these species and EMPF in horses regarding the age at clinical onset and progression of the disease as well as poor response to therapy.[25] No etiologic cause for fibrosis has been found in cats and dogs. In horses, donkeys and humans, pulmonary fibrosis was associated with EHV-5, AHV-4/5 and different gamma herpesviruses respectively.[37] Epstein-Barr Virus (EBV), cytomegalovirus, human herpesvirus (HHV) -7 and -8 were found in patients with idiopathic pulmonary fibrosis (IPF) in nearly 100% of diseased lungs compared with 40% of healthy patients.[38] Virus factors (latent versus lytic infection, expressed viral genes and chemokines), lung immunology (pre-existing TH2 environment, aged immune response), host factors (natural versus aberrant host, aged lung) and additional injury (co-infections, toxic injury) influence the development of pulmonary fibrosis.[25] Trigger factors of fibrosis are poorly understood. Epithelial cell injury, abnormal fibroproliferation, inflammation, deposition of extracellular matrix substances and viruses as co-factors for initiation, promoting or exacerbating pulmonary fibrosis are described.[39,40] Although recovery from severe pulmonary damage was recently demonstrated in an equine model of perilla mint ketone-induced acute lung injury,[41] fibrosis is still characterized as a chronic, progressive, irreversible pathologic process with poor prognosis.[41]

Pathophysiology of EMPF is largely unknown. A recent study showed that epithelial cells lining the mucosa of nasal and tracheal explants and primary equine respiratory epithelial cells were not susceptible to EHV-5 infection. In contrast, EHV-5-positive cell clusters were observed after infection of lung explants with EHV-5.[20] In the same study 10% of the inoculated T and B lymphocytes synthesized intracellular virus antigen post infection, indicating spreading the of the virus via cell-to-cell transfer.[20] The authors concluded based on their results that a hypothetical model of EHV5 pathogenesis in horses starts with transportation of virions by the mucociliary escalator towards the tonsillar crypts in the nasopharynx. EHV-5 then directly infects lymphocytes situated in the lymphoid follicles. From there the infected lymphocytes reach the blood stream directly or via lymph vessels and draining lymph nodes. The virus spreads by cell to cell transfer to adjacent lymphocytes in the lymphoid follicles or draining lymph nodes. EHV-5 infected lymphocytes might either undergo apoptosis or survive and provide a life-long reservoir for EHV-5. Via blood flow or via lymphocyte-homing, EHV-5 infected lymphocytes (re)routes to different parts of the respiratory tract, e.g. nasal cavities, trachea or lungs. Amplification of the infection in epithelial cells, shedding of high viral loads in respiratory secretions and cell-cell transfer to neighboring alveolar cells may ensue. The onset of fibrosis may be triggered by viral replication and host-specific predisposing factors.[20]

Acyclovir acts as a nucleoside analog that substitutes guanosine triphosphate in DNA synthesis. Due to its mode of action, the antiviral effect occurs only during replication of the virus. It is selectively toxic to herpesviruses due to its higher affinity for viral thymidine kinase (TK) and DNA polymerase compared with host TK and cellular polymerase.[42] Early treatment of neuropathogenic EHV-1 infection in horses with valacyclovir has shown decreased viral replication, viral shedding and viremia[43]. Similar action is supposed to occur in horses with EHV-5 infection. Acyclovir is less effective against equine herpesviruses than against human herpes viruses such as herpes simplex virus and has a poor oral bioavailability in horses. Valacyclovir, the prodrug of acyclovir, shows better oral bioavailability data.[44] Still, the best studied antiherpetic drug for EHVs due to its lower costs, high safety and availability remains acyclovir.[45] Based on pharmacokinetics and empiric values, various therapeutic protocols with valacyclovir are described. A loading-dose regime of 27 mg/kg orally every eight hours for the first two days followed by a maintenance dose regimen of 18 mg/kg orally every 12 hours can be used as a guideline.[44] In the presented case series, antiviral therapy was not effective but successful treatment with valacyclovir has been reported in a horse in the past.[11] A recent study has shown no short-term effect of valacyclovir on EHV-5 viral load in EMPF affected horses.[45] No association between EHV-5 viral load in the lungs ante mortem and survival time was found in 14 cases.[2] Treatment with antivirals in 7/14 horses (6 with valacyclovir, 1 with acyclovir) was also not associated with short-time survival.[2] This is not surprising, as fibrosis is induced during the latency of the virus, not during the active replicating phase when valacyclovir exerts its effect. The long-term effect of antivirals, however is unknown and needs to be investigated. Corticos-

teroids have also been proposed as treatment for EMPF and short-term mortality was reduced in 8/14 horses treated with corticosteroids compared to six horses without corticosteroid therapy.[2]

Clinical signs and findings in BALF and TA cytology can be similar to common chronic respiratory diseases, especially equine asthma syndrome (recurrent airway obstruction (RAO), inflammatory airway disease (IAD), chronic obstructive bronchitis (COB).[46] In some cases pyrexia, weight loss, tachypnea and severe dyspnea are present which can help distinguish equine asthma syndrome from EMPF.[26] On blood analysis, a marked inflammatory response with neutrophilia and hyperfibrinogenemia is common in cases with EMPF.[12] Radiographs show an interstitial to nodular pulmonary pattern.[26] BALF and TA cytology and culture is helpful to rule out fungal, bacterial, and silicate-induced granulomatous interstitial lung disease but it is often not possible to distinguish equine asthma syndrome from acute EMPF. BALF can be submitted for PCR and many reported EMPF cases were positive for EHV-5; however a negative result does not fully rule out EMPF.[27] Thoracic radiographs and ultrasound are helpful in distinguishing equine asthma from EMPF. Lung biopsies allows a definite ante mortem diagnosis. Histologically, multiple, well-demarcated nodular regions of pulmonary interstitial fibrosis with mixed inflammatory cell infiltration and a positive EHV-5 PCR result are reported as gold standard.[26] Overall, prognosis for EMPF remains poor. In the recent case series of 14 horses the median short- and long-time survival were 18 and 27.5 days, respectively[2] with only one horse being alive more than three years after initial diagnosis. These findings are comparable to the outcome of the three horses in the presented case series. One horse survived nine months after initial diagnosis, the other two cases were euthanized after 23 days and 45 days, respectively. Severity of thoracic radiographic changes and EHV-5 viral load measured by quantitative qPCR in lung tissue at presentation did not correlate with survival time. The administration of corticosteroids had a positive effect on short-term survival.[2]

In conclusion, EMPF should be considered as a differential diagnosis for acute or chronic lower respiratory disease. Thoracic radiographs and thoracic ultrasound should be performed in cases with suspected equine asthma syndrome that do not improve with adequate therapy. A lack of EHV-5 in BAL fluid or on PCR from lung biopsy samples does not rule out EMPF. Other equid gamma herpesviruses like AHV-5 should be considered when investigating the cause of EMPF. If results from EHV-5 specific PCR of BAL fluid or lung tissue are negative, a panherpes nested PCR (detection of all known herpesviruses) with subsequent sequencing to determine other potentially involved herpesviruses should be pursued.

Fibrose pulmonaire multinodulaire équine: une série de cas mettant en évidence diverses manifestations cliniques

Cette série de cas décrit trois cas de fibrose pulmonaire multinodulaire équine (EMPF) diagnostiqués à la Clinique de médecine interne équine de l'Université de Zurich entre 2012 et 2017. Des informations actuelles sur l'étiologie et les options de traitement sont présentées. Deux chevaux présentaient de légers signes de maladie chronique des voies respiratoires inférieures et un cheval présentait des signes aigus de maladie, notamment des pics de fièvre récurrents et une tachypnée. Le diagnostic a été obtenu grâce à un examen physique, des résultats radiographiques et des tests PCR pour les virus herpès équins (EHV) du liquide de lavage broncho-alvéolaire (BAL) ou du tissu pulmonaire obtenus par biopsie. Tous les chevaux ont été euthanasiés en raison d'une détérioration continue après une tentative de traitement. L'examen histologique post mortem du tissu pulmonaire a montré une fibrose multifocale diffuse à confluente sévère dans deux cas et chez un cheval un type de fibrose nodulaire discret. La PCR par Panherpes a révélé la pré-

Una serie di casi che illustrano il ruolo dei diversi Herpesvirus gamma nella fibrosi polmonare multinodulare equina

In questo studio sono illustrati tre casi di fibrosi polmonare multinodulare equina (EMPF) diagnosticati tra il 2012 e il 2017 presso la clinica di medicina interna equina dell'Università di Zurigo. Le informazioni attuali sull'eziologia e sulle possibilità di trattamento sono descritte. Due cavalli mostravano lievi segni di una malattia cronica delle basse vie respiratorie e un cavallo aveva dei segni acuti della malattia tra cui picchi ricorrenti di febbre e tachipnea. La diagnosi è stata determinata via un esame fisico, i risultati radiografici e un test PCR per gli herpesvirus equini (EHV) del liquido di lavaggio broncoalveolare (BAL) o del tessuto polmonare ottenuto mediante biopsia. Tutti i cavalli sono stati eutanasiati a causa del continuo deterioramento del loro stato dopo il tentativo di trattamento. L'esame istologico post mortem del tessuto polmonare ha evidenziato in due casi una grave fibrosi multifocale diffusa o a confluenza e in un caso un discreto modello di fibrosi. La pan-herpes nested PCR ha rilevato la presenza

sence d'ADN de virus herpès équin 5 (EHV-5) dans le tissu pulmonaire d›un cheval alors que chez deux autres chevaux, le virus de l›herpès asinien 5 (AHV-5) a été détecté. L'EMPF doit être considéré comme un diagnostic différentiel chez les chevaux souffrant d'une maladie respiratoire aiguë et chronique, y compris les chevaux ne répondant pas au traitement de l'asthme équin.

Mots-clés: virus herpès asinien 5; l'virus herpès équin 5; cheval; pneumonie interstitielle; fibrose pulmonaire

dell'herpesvirus equino 5 (EHV-5) DNA nel tessuto polmonare in un cavallo mentre negli altri due ha rilevato il virus dell'herpes asinino 5 (AHV-5). La EMPF deve essere quindi presa in considerazione per la diagnosi differenziata nei cavalli che soffrono di una malattia acuta e cronica delle vie respiratorie compresi i casi di cavalli che non rispondono ad un trattamento per l'asma equina.

Parole chiave: virus dell'herpes asinino 5; herpesvirus equino 5; cavalli; polmonite interstiziale; fibrosi polmonare

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