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Variability of Fungal Detection in the Airways of Horses With and Without Clinical Signs, According to the Sample Site and Analytical Methods

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Abstract

Fungi detection in equine airways may be performed on either tracheal wash (TW) or bronchoalveolar lavage fluid (BALF) by either cytology or culture. However, method comparisons are sparse. The objective was to determine the prevalence of fungi in airways of horses with or without respiratory clinical signs, according to the sample site and laboratory methodology. Sixty-two adult horses, investigated in the field or referred for respiratory disease, were included.TW and BALF were collected from each lung separately through a videoendoscope. Fungi were detected by cytology and culture. Overall prevalence of fungi was of 91.9% in TW and 37.1% in BALF. Fungi were positively cultured from 82.3% TW and 20.9% BALF. Fungal elements were observed by cytology in 69.4% TW and 22.6% BALF. Prevalence of fungi was not significantly different between horses with or without clinical signs. In 50%of horses, the same fungi were detected in both TW and hay, but fungi detected in BALF and hay did not correspond for any horse. Poor agreement was found between TW and BALF and between culture and cytology (Cohen's kappa coefficient (κ) < .20). Moderate agreement was found between cytology of left/right lungs (κ = .47). The prevalence of fungi by cytology on pooled BALF was significantly different (p = .023) than on combined left + right BALF. A high prevalence of fungi was detected in the lower respiratory tract of horses, particularly in the TW. Hay might not be the primary source of fungi of the lower respiratory tract of horses.

INTRODUCTION

Interest in fungi in the respiratory tract of horses is increasing substantially among equine clinicians, although publications on the subject are sparse. Fungal elements are commonly present in equine airways (up to 55% of tracheal wash (TW) by culture, and 79% by cytology), and a significantly increased risk of mild-moderate equine asthma (mEA) was found in their presence (Dauvillier et al., 2019). Fungi may also play a role in the pathogenesis of severe equine asthma (sEA) and successfully elicited pulmonary disease when experimentally exposed to asymptomatic sEA horses, similarly to hay dust (McGORUM et al., 1993; Pirie et al., 2003, 2002). Fungi are ubiquitous in the environment of horses, in hay, soil, and bedding (Stewart and Cuming, 2015). Respirable dust in the environment may contain a variety of organic and inorganic particles, including fungi, molds and their constituents, and particles of small size (< 5 μm (Cha and Costa, 2017)) can be inhaled to the alveolar level. Dry hay is one of the main sources of respirable dust in stabled horses and has been associated with a greater than two-fold increase in odds of finding fungal elements in the TW (Dauvillier et al., 2019; Olave et al., 2021). Indeed, moldy hay is routinely used as an exacerbation tool for investigation of sEA (Ivester et al., 2014; Kang et al., 2022; McGORUM et al., 1993). However, to our knowledge, no comparison of the mycological composition of hay to fungi retrieved from the respiratory tract of horses has been performed to date.

Identification of the most reliable sample and analysis modalities for the detection of fungi in the respiratory tract of horses is necessary. While poor agreement was observed between TW culture and cytology for the detection of fungi (Dauvillier et al., 2019), to our knowledge there has been no similar comparison with bronchoalveolar lavage fluid (BALF), or between TW and BALF. Poor correlation between TW and BALF cytology for the diagnosis of respiratory inflammation has been shown previously (Malikides et al., 2003). Samples from pooled BALF ($BALF_p$) and individual (left/right) lungs have been compared for the analysis of inflammatory cells and hemosiderophages, with a fair to moderate agreement observed between individual lungs for the diagnosis of mEA and/or exercise-induced pulmonary hemorrhage (EIPH) (Depecker et al., 2014; Hermange et al., 2019). In addition, excellent correlation was found between pooled BAL samples and samples from both lungs combined for the diagnosis of inflammation (Hermange et al., 2019). Similar comparisons would be valuable for characterization of fungi in the respiratory tract of horses.

In order to characterize fungal detection and identification from airway samples of horses, our objectives were to 1) determine the prevalence of fungi in the respiratory tract of horses with or without prior clinical suspicion of respiratory disease, and 2) compare the methodologies (fungal culture and cytology) as well as sample sites (TW and BALF from both lungs).

MATERIALS AND METHODS

Horses and Study Design

The study included 30 Standardbred racehorses, sampled in the field between October and December 2020, and 32 horses referred to the hospital between February and December 2021. Inclusion criteria were respectively to be in active training (field), or to be referred for decreased performance and/or respiratory disease. Data regarding housing conditions (indoor/outdoor), bedding (straw, wood shavings), hay forage (dry/soaked/steamed), activity (training/resting) and duration since last training, occurrence of poor performance or exercise intolerance, as well as reported respiratory clinical signs (cough, dyspnea, nasal discharge, noise during exercise, increased respiratory effort at rest, epistaxis), were collected.

Each horse was submitted to a thorough clinical examination by an ECEIM diplomate or resident. If observed, the presence of cough, nasal discharge, nostril flaring at rest, or dyspnea was recorded. Rebreathing pulmonary auscultation was performed and abnormal pulmonary (wheezes/crackles) or tracheal sounds, induction of cough, and delayed recovery following removal of the rebreathing bag were recorded. Venous blood samples were collected for hematology, and additional biochemical analysis when judged clinically necessary.

Respiratory sampling

Horses that were not racing or competing within 10 days following the sampling were sedated with detomidine (10 μg/kg IV, Sédomidine, Audevard), with the addition of butorphanol (10–20 μg/kg IV, Torphadine, Dechra) when needed. A nose twitch was used when necessary. For horses racing

within 4 to 10 days following investigation, the procedures were achieved with a nose twitch only, because of drug testing considerations.

Tracheal wash

A 120 cm long videoendoscope (AOHUA off-site, OLYMPUS in-house) was passed through one of the nostrils into the pharynx. Pharyngitis was scored using a scale of 1–4.(Raker and Boles, 1978) The endoscope was then inserted into the proximal trachea, and tracheal mucus was scored using a scale of 0–5 (Gerber et al., 2010). A single-use double-lumen sterile catheter was passed through the endoscope channel, and 40 mL of sterile saline solution (.9% NaCl) was infused. The endoscope was then advanced down the trachea to reach and retrieve the pool of fluid. The aspirated fluid was divided between a sterile dry tube which was submitted for microbiology (bacteriology and fungal culture), and a tube containing EDTA which was submitted for cytological analysis.

Bronchoalveolar lavage

Where clinical signs were marked (labored breathing, severe bronchospasm, persistent cough), intravenous spasmolytics (scopolamine [.16 mg/kg] and metamizole [20 mg/kg] IV (Estocelan, Boehringer Ingelheim)) were administered prior to bronchoalveolar lavage. The BAL was performed using a flexible 320 cm long videoendoscope (AOHUA off-site, OLYMPUS in-house). The endoscope was inserted into the left or right main bronchus until wedged. A total of 500 mL (two boluses of 250 mL each) of warmed (37°C) sterile isotonic saline solution was instilled through the endoscope biopsy channel, using 60-mL pre-filled syringes. After each bolus, the liquid was aspirated with the same syringes, and the first 20 mL corresponding to the endoscope channel volume that did not reach the lung was discarded. After lavage of the first lung, the endoscope channel was cleaned with a 60 mL bolus of sterile isotonic saline. The endoscope was then relocated to the carina, introduced in the contralateral lung and the lavage procedure was repeated.

The volume of liquid collected and macroscopic assessment (color, turbidity, and presence of foam) were recorded. A proportional volume of BAL from each lung was pooled in a jar. Left, right and pooled samples were then placed in a tube containing EDTA, which was submitted for cytological examination, and another aliquot of pooled samples was also placed in a sterile tube, which was submitted for bacterial and fungal culture. All samples were kept chilled until handled by the laboratory, and all analyses were performed within 24 hours of sampling.

Endoscopes were sterilized between each horse by flushing the channels and soaking the endoscopes in disinfectant diluted as a .5% solution (Hexanios G + R, Anios Laboratories) as per manufacturer’s recommendations.

Microbiology

Bacteriological examinations were performed as previously described(Richard and Maillard, 2009) and consisted of direct isolation on selective and non-selective media, Gram staining and plate inoculation after dilution for counting (Richard and Maillard, 2009). Mycology testing was performed by direct isolation on selective media (Online Ressource 1). Samples were incubated for up to 6 days, and identification was performed by biochemical identification (standard API kits, Biomerieux).

In order to rule out extrinsic contamination by the equipment, videoendoscopes were tested for fungal contamination. Sampling of each endoscope was performed twice (two months apart) throughout the study. Briefly, the tip of the disinfected videoendoscopes was placed in an empty, sterile, plastic jar. Two 60-mL syringes pre-filled with sterile saline were infused through the biopsy channel into the sterile jar, with the tip of the scope dipping in the solution. The infused solution was then aspirated through the biopsy channel and placed in a sterile tube for mycological culture.

Cytology

Two hundred microliters of fluid from TW and BALF (left, right and pooled) were cytocentrifuged (80 *g*, 10 min) and stained with May-Grünwald-Giemsa (MGG). A differential cell count was performed on 300 cells (Richard et al., 2010) and the number of each cell type was recorded as a percentage of total nucleated cells, excluding epithelial cells. The presence/absence of fungal particles (spores and hyphae, either extracellular or phagocyted) and bacteria was screened over the entire cytological slide and qualitatively documented (Online Ressource 2).

Hay processing and analysis

Hay was sampled from each yard for horses examined in the field. One kilogram of hay was collected and dried overnight at 37°C. The next day, hay was centrifugally ground (ZM200 Ultra Centrifugal Mill, Retsch). Total flora was analyzed after mixing 20g of ground hay with 180 mL of .1% buffered peptone water and 10 − 1 to 10 − 6 dilutions. Samples were incubated at 37°C and microbial quantification was performed on days 2 and 5.

For mycological analysis (Online Ressource 1), 1 mL of the previously described diluted solution was spread on an Oxytetracycline-glucose-yeast extract (OGA) agar and incubated at 25°C. Colonies were then isolated on Sabouraud agar, and incubated at 25°C for 5 days before identification, which was performed by direct observation and isolation on slides after coloration with .4% Tryptan blue.

Case definition

Bacterial culture was considered clinically significant if $> 10^3$ colony-forming units (CFU) /mL were counted, and characterized as polymorphic flora when more than 3 genera were identified (Doubli-Bounoua et al., 2016; Richard et al., 2010). Fungal culture was classified as positive when one or

more colonies were observed (and identified). Cytology was classified as positive for fungal elements when at least one fungal element (spore, hyphae), either intra- or extracellular, was observed on the slide.

Statistical analysis

Normality of distribution of continuous variables was evaluated using the Shapiro–Wilk W test. Categorical variables were summarized using number and percent of observations in each category. Comparison of prevalence between groups was performed with Yates' X^2 test. All data were then gathered for comparative analysis of sample sites and methods. Comparison between sample sites (TW, BAL_P, BAL_L, BAL_R) and between methods (culture *vs.* cytology) was assessed using the McNemar Test with continuity correction. Agreement between sample sites and methods was assessed with Cohen's kappa coefficient (κ). Values of $P < .05$ were considered statistically significant.

RESULTS

Horses without prior clinical suspicion, evaluated at the yard

Signalment

The 30 Standardbred racehorses (33.3% (n = 10) geldings, 40.0% (n = 12) mares and 26.7% (n = 8) males) were aged 2–7 years (mean 4.2 ± 1.7 yo). Horses were located in 3 different training yards and were either in active training (80.0% (n = 24)) or off-work for 2–3 weeks (20.0% (n = 6)) due to poor performance. Sixteen (53.3%) horses were kept in pasture, and 46.7% of (n = 14) horses were housed overnight in stalls with straw bedding, except one horse on wood shavings. All horses were fed dry hay. Respiratory clinical signs reported by trainers included serous nasal discharge (30.0%, n = 9), epistaxis (3.3%, n = 1) and poor-performance (33.3%, n = 10). On rebreathing pulmonary auscultation, mild crackles (50.0%, n = 15) and wheezes (6.7%, n = 2) were audible. None of the horses presented neither cough or dyspnea. Seventeen (56.7%) horses presented with a pharyngeal grade $\geq$ 3/4. Twelve (40%) horses presented with a tracheal mucus score $\geq$ 2/5.

Diagnosis

Six (20.0%) horses were diagnosed with mEA, 7 (23.3%) horses were diagnosed with EIPH without associated pulmonary inflammation based on BALF cytology, and 7 (23,3%) horses were diagnosed with concomitant EIPH and mEA. Ten (33,3%) horses were considered free from respiratory disease.

TW / BALF microbiology

Eight (26.7%) horses presented a positive bacterial culture on the TW, of which 25.0% (n = 2) were polymorphic flora without predominating bacteria identified, 12.5% (n = 1) showed low level of contamination (< 10^3 CFU/mL), and 62.5% (n = 5) had $\geq 10^3$ CFU/mL, consistent with active bacterial infection (Online Ressource 3).

Fungal culture from the videoendoscopes was negative for all samples. Fungal culture was positive for 93.3% (n = 28) of TW and 13.3% (n = 4) of $BALF_P$. Eight fungal genera were identified in TW (Table 1), *Aspergillus* (67.9%, n = 19) and *Penicillium* (60.7%, n = 17) being mostly represented either alone or concomitant with other fungi (Fig. 1).

Table 1
Less commonly identified fungi by culture (n = 62)

	TW		$BALF_P$	
Fungi	Number of positive cases	Number of colonies/fungi (min-max)	Number of positive cases	Number of colonies/fungi (min-max)
Scopulariospsis spp.	2	1–2		
Beauveria spp.	1	1		
Alternaria spp.	5	1–3		
Mucor spp.	3	1		
Chrysosporium spp.	14	1		
Cladosporium spp.	6	1–3		
Candida guillermondii	1	1		
Acremonium spp.	1	1		
Scedosporium spp.	2	1–2		
Geotrichum spp.	1	2		
Paecilomyces spp.	1	1		
Aureobasidium pullulans			1	1

TW: Tracheal Wash; $BALF_P$: pooled Bronchoalveolar Lavage Fluid

Fungi identified in $BALF_P$ included *Penicillium* (50.0%, n = 2), *Aspergillus nidulans* (25.0%, n = 1) and *Aureobasidium pullulans* (25.0%, n = 1). All 4 horses with a positive $BALF_P$ fungal culture also exhibited a positive fungal culture on TW, with the same fungi found in both samples for 50.0% (n = 2) of horses (Table 2).

Table 2: Fungi identified from horses with a positive BALFP culture

Case number	9		17		18		21		33		39		41		46		49		50		53		59		60	
Fungi	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P	TW	BAL_P
Aspergilllus niger																										
A. fumigatus																										
A. glaucus																										
A. nidulans																										
A. versicolor																										
A. flavus																										
Penicillium spp.																										
Aureobasidium spp.																										
Chrysosporium spp.																										
Candida spp.																										
Mucor spp.																										

TW: Tracheal Wash ; BALFP : pooled Bronchoalveolar Lavage Fluid

TW / BALF cytology

Fungal elements were detected on TW cytology for 73.3% (n=22) of horses, of which one TW fungal culture was negative. One the other hand, 25.0% (n=7) of horses with a positive TW fungal culture showed no fungal element on TW cytology (Table 3; Online Ressource 4). Spores and hyphae were mainly found simultaneously in the same TW sample. Overall, phagocytosis was observed in 63.6% (n=14) of TW samples, with spores more frequently phagocyted by macrophages than hyphae (Table 4).

Table 3: Prevalence of positive fungal culture and cytology depending on sample sites

	Horses in field (n= 30)	**Horses referred (n= 32)**	**P-value In field *vs.* referred**	**Total (n= 62)**
Age	2-7 (4,2 1,7)	2-25 (11 4,7)	-	2-25 (7,4 4,8)
	Fungal culture			
TW	28 (93.3%)	23 (71.9%)	.06	51 (82.3%)
BAL_P	4 (13.3%)	9 (28.1%)	.26	13 (20.9%)
	Fungal elements on cytology			
TW	22 (73.3%)	21 (65.6%)	.70	43 (69.4%)
BAL_R	8 (26.7%)	8 (25.0%)	.89	16 (25.8%)
BAL_L	7 (23.3%)	8 (25.0%)	.89	15 (24.2%)
BAL_P	7 (23.3%)	7 (21.9%)	.87	14 (22.6%)

TW: Tracheal Wash; BALF : pooled Bronchoalveolar Lavage Fluid, $_P$: pooled, $_L$: left, $_R$: right. P-value < 0.05 was significative

Fungal elements were identified by cytology in 26.7% (n = 8) of right BALF ($BALF_R$), 23.3% (n = 7) of left BALF ($BALF_L$), and 23.3% (n = 7) of $BALF_P$. One horse for which the $BALF_P$ was positive on cytological examination, exhibited a negative TW cytology (Online Ressource 4). In the $BALF_R$, spores and hyphae were rarely found simultaneously in the same sample. Overall, phagocytosis was observed in 62.5% (n = 5) of $BALF_R$ samples, with spores more frequently phagocyted by macrophages than hyphae (Table 4). In the $BALF_L$, all samples contained spores, which were frequently phagocyted by macrophages, and no hyphae were observed. In the $BALF_P$, spores and hyphae were rarely found simultaneously in the same sample, but were frequently phagocyted by macrophages. Overall, phagocytosis was observed in 57.1% (n = 4) of $BALF_P$ samples, with spores more frequently phagocyted by macrophages than hyphae (Table 4).

Table 4
Distribution and phagocytosis of fungal elements depending on sample sites

	Spores (%)	Hyphae (%)	Spores + Hyphae (%)
Horses in field (n = 30)			
TW	18/22 (81.8%)	17/22 (77.2%)	12/22 (54.5%)
Phagocyted	13/18 (72.2%)	7/17 (41.2%)	
BAL_P	7/7 (100%)	2/7 (28.6%)	2/7 (28.6%)
Phagocyted	6/7 (85.7%)	2/2 (100%)	
BAL_L	7/7 (100%)	0	0
Phagocyted	6/7 (85.7%)	0	
BAL_R	5/8 (62.5%)	6/8 (75.0%)	3/8 (37.5%)
Phagocyted	4/5 (80.0%)	2/8 (33.3%)	
Horses referred (n = 32)			
TW	18/21 (85.7%)	16/21 (76.2%)	12/21 (57.1%)
Phagocyted	15/18 (83.3%)	9/16 (56.3%)	
BAL_P	7/7 (100%)	2/7 (28.6%)	2/7 (28.6%)
Phagocyted	7/7 (100%)	1/2 (50.0%)	
BAL_L	8/8 (100%)	2/8 (25.0%)	2/8 (25.0%)
Phagocyted	8/8 (100%)	2/2 (100%)	
BAL_R	7/7 (100%)	4/7 (57.1%)	4/7 (57.1)
Phagocyted	7/7 (100%)	2/5 (50.0%)	

TW: Tracheal Wash ; BALF : Bronchoalveolar Lavage Fluid, $_P$: pooled, $_L$: left, $_R$: right

Hay mycology

There was high variability in both quantity and identification of yeasts and molds retrieved from hay depending on yards and hay bales. Furthermore, only a few fungi found in hay were also cultured from the corresponding respiratory samples (Table 5). Indeed, while *Aspergillus* was identified from the TW of 67.9% (19/28) of horses with a positive fungal culture, it was identified from 25.0% (1/4) of hay samples only (Yard 3). On the other hand, while *Penicillium* was identified from the TW of 60.7% (17/28) of horses with a positive fungal culture, it was identified from 75.0% (3/4) of hay samples. None of the fungi identified in $BALF_P$ were found in the hay for any individual horse.

Table 5
Results of fungal culture results from hay and respiratory samples

	Yard 1			Yard 2		Yard 3	
	Haybale 1	Haybale 2	TW and $BALF_P$ samples	Haybale	TW and $BALF_P$ samples	Haybale	TW and $BALF_P$ samples
Quantification (CFU/g)	71000 (53000 yeast + 8000 molds)	4300 (1300 yeast + 3000 molds)	*Alternaria* (1 TW) Mucor (1 TW) Penicillium (7 TW)	8300 (3400 molds + 4900 yeast)	None	750 (750 molds + < 10 yeasts)	*A. niger* (1 TW) *Cladosporium* (1 TW) *Penicillium* (2 TW)
Species identified	*Rhodotorula* *Alternaria* *Mucor* *Penicillium*	White yeast *Alternaria* *Mucor* *Penicillium*		*Cladosporium* *Ulocladium* *Fusarium* Other molds		*A. niger* *Mucor* *Cladosporium* *Chrysosporium* *Penicillium*	

TW: Tracheal Wash; *$BALF_P$: pooled Bronchoalveolar Lavage Fluid* ; A. : *Aspergillus*

Horses referred to the hospital for respiratory clinical signs

Signalment

The 32 horses (46.9% (n = 15) geldings, 46.9% (n = 15) mares and 6.3% (n = 2) males) were aged 2–25 years (mean 10.0 ± 4,8 yo). Breeds included saddlebred (n = 14), mixed breeds (n = 5), ponies (n = 4), Standarbred (n = 4), draft horses (n = 3), “masked for review” (n = 1) and “masked for review” (n = 1). Activities ranged from retired or leisure horses to high-level sport horses and racehorses in training. Nineteen horses (59.4%) were housed in stalls overnight, with 26.3% (n = 5) horses on wood shavings and 73.7% (n = 14) on straw bedding. The other horses were kept in pasture. Seventeen (53.1%) horses were fed dry hay, seven (21.9%) horses were fed soaked hay, one (3.1%) horse received haylage, four (12.5%) horses received steamed

hay with the use of a commercialized steamer (Haygain®), and three (9.4%) horses were fed "homemade" steamed hay. Respiratory clinical signs included serous to seromucous nasal discharge (59.4%, n = 19), dyspnea (59.4%, n = 19), nasal flaring (31.3%, n = 10), chronic cough at rest and/or during exercise (71.9%, n = 23), poor-performance (15.6%, n = 5) or exercise intolerance (50.0%, n = 16) and epistaxis (3.1%, n = 1). On rebreathing pulmonary auscultation, mild to severe crackles (53.1%, n = 17) and wheezes (18.8%, n = 6) were audible. Cough was elicited in 28.1% (n = 9) of horses, and recovery time following the test was increased in 59.4% (n = 19) of horses. Four (12.5%) horses presented with a pharyngeal grade $\geq$ 3/4. Fourteen (50%, 14/28) leisure or sport horses presented with a tracheal mucus score $\geq$ 3/5, and one racehorse (3.1%, 1/4) presented with a tracheal mucus score $\geq$ 2/5.

Diagnosis

Thirteen (40.6%) horses were diagnosed with mEA, 14 (43.8%) were diagnosed with sEA and 2 (6.3%) horses were diagnosed with EIPH without associated pulmonary inflammation based on BALF cytology. Other diagnosis included eosinophilic interstitial pneumonia (3.1%, n = 1) based on BALF cytology and thoracic radiography, as well as subclinical bacterial pulmonary infection (3.1%, n = 1) and pharyngitis (3.1%, n = 1) without associated pulmonary inflammation based on BALF cytology.

TW/BALF microbiology

Sixteen (50.0%) horses presented with a positive bacterial culture on the TW, of which 43.8% (n = 7) were polymorphic flora without predominating bacteria identified, 12.5% (n = 2) showed a low level of contamination (< 10^3 CFU/mL), and 43.8% (n = 7) had $\geq 10^3$ CFU/mL consistent with active bacterial infection (Online Ressource 3).

Fungal culture was positive for 71.9% (n = 23) of TW and 28.1% (n = 9) of $BALF_P$. Eleven fungal genera were identified in TW (Table 1); *Aspergillus* (47.8%, n = 11) and *Penicillium* (52.2%, n = 12) were mostly represented either alone or concomitant with other fungi (Fig. 1). Fungi identified in $BALF_P$ included *Penicillium* (33.3%, n = 3) and *Aspergillus* (77.8%, n = 7), either alone or concomitantly (Fig. 1). All horses with a positive $BALF_P$ fungal culture except one also exhibited a positive fungal culture on TW, with the same fungi found in both samples for 55.6% (5/9) of horses (Table 2).

TW/BALF cytology

Fungal elements were detected on TW cytology for 65.6% (n = 21) of horses, of which 28.6% (n = 6) TW fungal culture was negative. On the other hand, 30.4% (n = 7) of horses with a positive TW fungal culture showed no fungal element on TW cytology (Table 3; Online Ressource 4). Spores and hyphae were mainly found simultaneously in the same TW sample. Overall, phagocytosis was observed in 85.7% (n = 18) of TW samples, with spores more frequently phagocyted by macrophages than hyphae (Table 4).

Fungal elements were detected in 21.9% (n = 7) of $BALF_R$, 25.0% (n = 8) of $BALF_L$, and 21.9% (n = 7) of $BALF_P$. All horses with a positive $BALF_P$ cytology were also positive on TW cytology (Online Ressource 4). In the $BALF_R$, spores and hyphae were frequently found simultaneously in the same sample. Overall, phagocytosis was observed in 100% (n = 7) of $BALF_R$ samples, with spores more frequently phagocyted by macrophages than hyphae (Table 4). In the $BALF_L$, spores and hyphae were rarely found simultaneously in the same sample, and were all phagocyted by macrophages. In the $BALF_P$, spores and hyphae were rarely found simultaneously in the same sample. Overall, phagocytosis was observed in 100% (n = 7) of $BALF_P$ samples, with spores more frequently phagocyted by macrophages than hyphae (Table 4).

Comparison of methods and sample sites

Horses without prior clinical suspicion vs. horses referred to the hospital

The prevalence of fungi was not statistically different between groups of horses (Table 3). All 62 samples were gathered for further comparisons of sample sites and laboratory methods.

Fungal culture vs. cytology

Of the 62 samples, 91.9% (n = 57) of TW and 37.1% (n = 23) of $BALF_P$ were positive by fungal culture and/or cytology. In the TW, 58.1% (n = 36) of horses had a positive fungal culture and cytology, while 22.6% (n = 14) and 11.3% (n = 7) of horses respectively were positive by fungal culture or cytology only (κ = .11, 95% CI − .14 − .36), with no difference detected in prevalence (Fig. 2).

In the $BALF_P$, 6.5% (n = 4) of horses had a positive fungal culture and cytology, while 14.5% (n = 9) and 16.1% (n = 10) of horses respectively were positive by fungal culture or cytology only (κ = .10, 95% CI − .17 − .37), with no difference detected in prevalence (Fig. 2).

TW vs. BALFs

The proportion of samples positive by fungal culture differed significantly ($p < .001$) by sample sites, with 82.3% (n = 51) of TW samples and 20.9% (n = 13) of $BALF_P$ samples positive (Fig. 2). In addition, 19.4% (n = 12) of horses were positive by fungal culture in both TW and $BALF_P$, while 61.3% (n = 38) and 1.6% (n = 1) of horses respectively were positive in either TW or $BALF_P$ only (κ = .07, 95% CI − .03 − .17).

The proportion of TW samples positive by cytology (Table 3) differed significantly ($p < .001$) from all BAL sites. Similarly, the proportion of $BALF_P$ samples positive by cytology differed significantly ($p = .023$) from combined $BALF_L$ and $BALF_R$; while the proportion of positive samples by cytology were not significantly different between $BALF_L$ and $BALF_R$.

On cytology, 21.0% (n = 13) of horses were positive for fungal elements in both TW and $BALF_P$, while 48.4% (n = 30) and 1.6% (n = 1) of horses respectively were positive in either TW or $BALF_P$ only (κ = .18, 95% CI .04 − .31). Fourteen (22.6%) horses were positive in both TW and $BALF_L$, while 46.8% (n = 29) and 1.6% (n = 1) of horses respectively were positive in either TW or $BALF_L$ only (κ = .19, 95% CI .05 − .34). Eleven (17.7%) horses were positive in both TW and $BALF_R$, while 51.6% (n = 32) and 6.5% (n = 4) of horses respectively were positive in either TW or $BALF_R$ only (κ = .03, 95% CI .13 − .19).

On cytology, 14.5% (n = 9) horses exhibited fungi in both $BALF_L$ and $BALF_R$, while 9.7% (n = 6) and 9.7% (n = 6) of horses respectively were positive in either $BALF_L$ or $BALF_R$ only (κ = .47, 95% CI .23 – .73). All 14 horses (100%) positive in $BALF_P$ were also positive in $BALF_L$ and/or $BALF_R$, while 14.6% (7/48) of horses with negative $BALF_P$ cytology were positive in $BALF_L$ and/or $BALF_R$.

DISCUSSION

The current study characterized fungal detection and identification from airway samples of horses. A high proportion of samples were positive for fungi by both culture and cytology, with phagocytosis of fungal elements commonly observed. In addition, there was very little concordance between fungi identified in the respiratory tract and in the hay fed. Finally, high variability of distribution and type of fungal elements in sample sites, of phagocytosis, and between culture and cytology was observed.

Prevalence

The prevalence of fungal detection by cytology in the tracheal wash (69.4% [95% CI .5788-.8083]) in the current study was similar to previously described (79% [95% CI .7612-.8202]) (Dauvillier et al., 2019). In contrast, the prevalence of positive fungal culture in the tracheal wash (82.3% [95% CI .7275-.9177]), with a predominance of *Aspergillus* and *Penicillium*, was considerably higher than previously described (up to 55% [95% CI .5125-.5846]) (Dauvillier et al., 2019). No standardized method for mycological examination from respiratory samples has yet been established, and pre-analytical methodology differed between studies (Borman et al., 2010; Denning et al., 2014). In the current study, samples were transported under refrigerated conditions in sterile tubes, while temperature during transport was not mentioned in a previous study (Dauvillier et al., 2019), where Sabouraud agar tubes were used. Transport at room temperature in the mentioned study may have altered fungal growth if analysis was delayed, potentially leading to lower detection rates (Stewart and Cuming, 2015).

Interestingly, phagocytosis of fungal elements was observed in three-quarters of TW samples and most of BALF samples, with spores more often phagocyted than hyphae. Similarly to bacteria, finding fungal elements on cytology has been reported as more suggestive of true infection when phagocyted by neutrophils or macrophages (Bain, 1997; Cian et al., 2015). Additionally, while a small amount of degenerate neutrophils may be normally found in respiratory secretions due to normal clearance from the body (Bain, 1997), a larger amount of degenerate neutrophils has been considered a positive indicator of bacterial infection (Jocelyn et al., 2018). To our knowledge, there is to date no published investigation on degenerate neutrophils related to fungal infections. In the current study, only 3 horses (1 TW, 2 BAL_R) were identified with degenerate neutrophils, irrespectively to the fungal culture of the same sample.

A high prevalence of fungi was found in the current study, which was similar between horses with and without prior clinical suspicion of respiratory disease. Prevalence was higher in the TW than BALF, both by culture and cytology, and may be in favor of environmental contamination rather than true infection. Further studies are warranted in order to determine whether detecting fungi in the respiratory tract of horses is a risk factor for respiratory disease such as equine asthma, as being previously suggested (Dauvillier et al., 2019).

Samples and modalities

Hay is recognized as one of the primary sources of inhaled fungal particles in horses, and has been used as an initiating/exacerbating agent for eliciting severe equine asthma (Pirie et al., 2003). However, in the current study, only a few fungi found in hay fed were also cultured from corresponding respiratory samples. These findings suggest that hay might not have been the primary source of molds/yeasts detected from the lower respiratory tract of these horses. Testing straw from each yard for mold content, in addition to hay, would have been relevant but was not performed in the current study for financial reasons.

There was only slight agreement between fungal culture and cytology in both TW and BALF, while the prevalence was not different. This is consistent with previous studies showing poor agreement between fungal culture and cytology from the TW, with no correlation between a positive mycology and detection of fungal growth on cytology (Dauvillier et al., 2019).

To our knowledge, this is the first report of pooled BALF cytology from both lungs compared to each lung separately with a focus on fungi detection. There was only moderate agreement between individual (left and right lung) BALF samples. As a consequence, blind sampling of BALF, often leading to wedging of the BAL tube in the right caudal lung, may lead to false negative results for fungal elements on cytology, as also previously demonstrated for neutrophils and hemosiderophages (Couetil and Thompson, 2020; Depecker et al., 2014). In addition, fungal detection by cytology on pooled BALF was significantly different from combined samples from both lungs.

A very high proportion of samples in this study were found to be positive (91.9% in TW, 37.1% in BALF) by fungal culture and/or cytology, rendering questionable the clinical significance of the presence of fungi in the lower respiratory tract of horses, particularly when found in the TW. It has been suggested that to be cytologically significant, large numbers of fungi should be visualized (Stewart and Cuming, 2015). In the current study, only presence/absence (and not relative quantification) was considered, possibly contributing at least partially to the high proportion of positive samples observed. Establishment of a pathological cut-off, both for cytology and mycology, as previously described for bacterial infections and inflammatory diseases (Bain, 1997; Denning et al., 2014; Richard et al., 2010; Richard and Maillard, 2009), could help in differentiating between true fungal infection and environmental contamination.

Limitations

Seasonal variation has been found in the concentration of respirable particles in horse barns (Kutasi et al., 2011; Nardoni et al., 2005), with a significant influence on mEA subtypes based on BALF cytology (Hansen et al., 2018; Secombe et al., 2015). An effect of season on the presence of fungi in the respiratory tract of horses was not reported to the authors' knowledge, and horses were then indifferently sampled throughout the year in this study.

Sedation protocols were elected at the clinician's discretion based on the horse's clinical condition and competition constraints. Antitussive effects of sedatives on BALF cytology results have been previously studied, with no influence of butorphanol on BALF volume collected or neutrophil proportions (Westermann et al., 2005). While fungal elements were not included in these previous investigations, no significant influence of sedatives has currently been expected. Furthermore, the prevalence of detecting fungi by culture or cytology, both on TW and BALF, was not significantly different between referred cases and horses in the field, despite the systematic use of butorphanol for clinical horses and the absence of its use for non-clinical horses.

A total volume of 500 mL of sterile saline per lung was infused, according to recommendations for cytology (Robinson and Chairperson, 2001). However, studies have shown that increasing the volume of fluid instilled for BAL may lead to cytological variations (Couëtil et al., 2016; Orard et al., 2016). Additionally, dilution of sputum had a clear effect on yeast recovery in human patients, with fewer patients found positive with a more dilute sputum (Pashley et al., 2012). Whether infusing a smaller amount would have led to increased retrieval of fungal elements on cytology or culture from BALF is currently unknown.

While endoscopes were efficiently disinfected between horses, a possible BALF contamination due to the passage of the endoscope through the nasal cavities could not be ruled out. However, this was considered unlikely considering the low prevalence of fungi recorded from BALF by either culture or cytology, as well as the observation of horses with negative BALF / positive TW for fungal detection.

Delays from sampling to analysis is another concern. In the current study, all samples were systematically processed within 24 hours. All samples were kept refrigerated (+ 4°C) until processing, as recommended in order to prevent *in vitro* microbial proliferation and cellular degeneration.(Cian et al., 2015) In addition, signs of fungal proliferation (germinating spores, branching hyphae) were observed in only one sample.

Conclusion

Based on our findings, fungi are detected with a high prevalence in the lower respiratory tract of horses, and particularly in the TW (both by culture and cytology). Furthermore, there is significant variability and only poor agreement between sample sites as well as laboratory methods for fungal detection in equine airways. Further studies will determine whether the detection/identification of fungi in TW and/or BALF is clinically relevant and represents a risk factor for respiratory troubles such as equine asthma.

Declarations

ACKNOWLEDGMENTS

Preliminary results were presented to the ECEIM Virtual Congress 2021 (poster with oral communication, November 2021), the 11th ICEEP (poster, Uppsala, June 2022) and the BEVA Congress 2022 (oral presentation, Liverpool, September 2022).

STATEMENT OF ANIMAL ETHICS

The study was approved by the regional Ethic Committee for Clinical and Epidemiological Veterinary Research (CERVO-2020-3-V). A consent form was also signed by the owner.

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

L. Lemonnier contributed to study design, data collection and study execution, data analysis and interpretation, and preparation of the manuscript. A. Couroucé contributed to study design, data collection and study execution, data analysis and interpretation and preparation of the manuscript. M.

Cessans, P. Barbazanges and M.-P. Toquet contributed to data collection, study execution and data analysis. L. Petit contributed to data analysis. J.M. Cardwell contributed to study design and preparation of the manuscript. E.A. Richard contributed to study design, data analysis and interpretation, and preparation of the manuscript. All authors gave their final approval of the manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval

The study was approved by the regional Ethic Committee for Clinical and Epidemiological Veterinary Research (CERVO-2020-3-V).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

Not applicable

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Figures

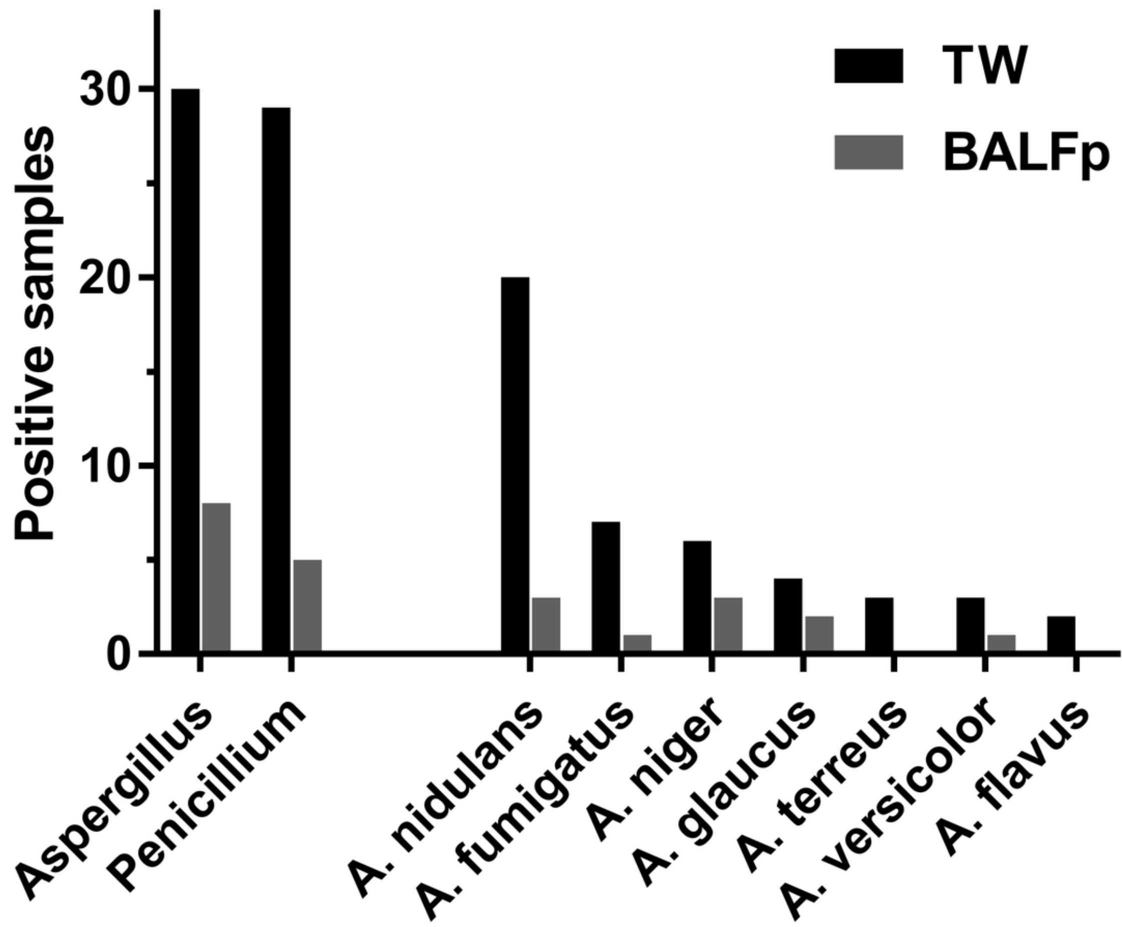

Figure 1

Most commonly identified fungi (*Aspergillus* and *Penicillium*) by culture in TW and $BAFL_P$ (n=62)

TW : Tracheal Wash ; $BALF_P$: pooled Bronchoalveolar Lavage Fluid , A. Aspergillus

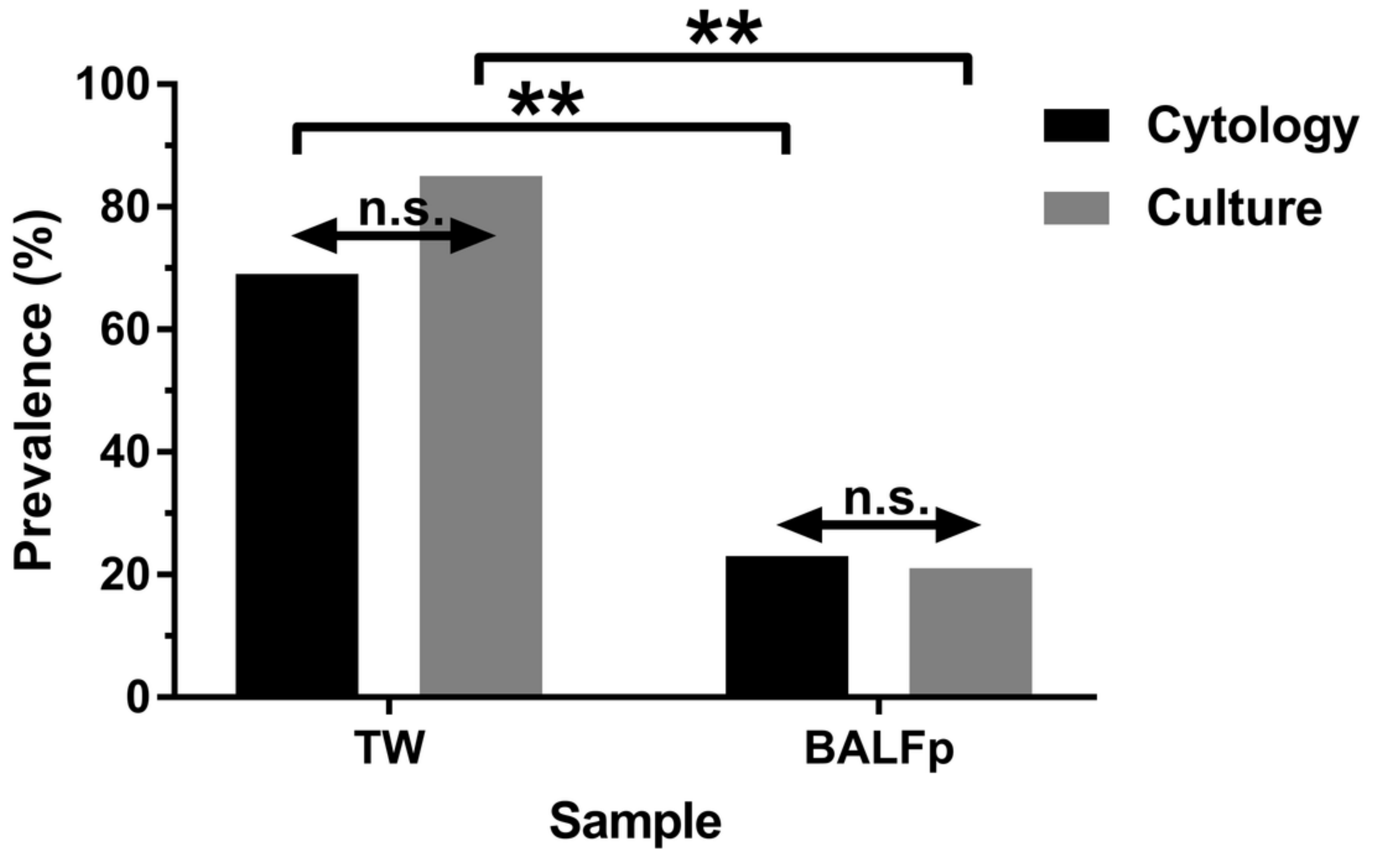

Figure 2

Prevalence of fungi (in percentage) depending on sampling sites and methods (cytology and culture)

*TW : Tracheal Wash ; $BALF_P$: pooled Bronchoalveolar Lavage Fluid ; n.s. : non significative ; **Significance was set at p < .05*

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