

# Association between fungal detection, airways inflammation and diagnosis of moderate to severe asthma in horses

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## Abstract

**Background:** Fungi are ubiquitous in horses' environment. Their contribution to the pathophysiology of severe asthma (SA) is acknowledged, while controversies remain for mild-moderate asthma (MA).

**Objectives:** We hypothesised that fungi are a risk factor for asthma. Our objective was to compare different combinations of analytical methods (cytology, culture) and sampling sites (tracheal wash (TW), bronchoalveolar lavage fluid (BALF)) in relation to clinical status (control, MA, SA).

**Study design:** Prospective cross-sectional study.

**Methods:** The study population included asymptomatic racing horses in the field and horses referred to the hospital for respiratory investigations. Fungi were detected by cytology and identified by mycology on TW and pooled BALF. Chi-square tests and multivariable logistic regressions were used for prevalence comparison between groups and association with clinical investigations.

**Results:** In total 155 horses (85 MA, 35 SA and 35 controls) were included in the study. The overall proportions of fungal detection in TW ranged from 45.7% to 89.4% among groups. The prevalence of fungal detection in BALF was significantly lower by cytology for SA (5.7%) than MA horses (23.6%) and significantly higher by culture for MA horses (31.8%) than controls (8.6%). Fungal detection by culture in BALF was significantly associated with high tracheal mucus score, high neutrophil proportions in BALF and diagnosis of MA.

**Main limitations:** Mycology was only performed in pooled BALF, and environment was not sampled.

**Conclusions:** Fungi were significantly more prevalent in the airways of MA horses than SA and/or controls. Fungal detection on TW, either by cytology or culture, was uninformative in a clinical context. Fungal detection by culture, but not cytology in BALF was associated with MA.

## KEYWORDS

equine asthma, fungi, horse, respiratory sampling

## 1 | INTRODUCTION

Equine asthma is a common cause of respiratory disease and poor performance. This term groups two distinctive phenotypes of inflammatory respiratory diseases: mild–moderate asthma (MA)—previously known as inflammatory airway disease (IAD), and severe asthma (SA)—previously known as recurrent airway obstruction (RAO).<sup>1</sup> The prevalence of SA has been estimated between 14% and 20% within the northern hemisphere,<sup>2,3</sup> and MA was identified in up to 66% of Standardbred racehorses presented for poor performance in France.<sup>4</sup>

Equine asthma, regardless of its severity, has common clinical presentations (chronic cough, excess mucus, poor performance) but a wide heterogeneity in terms of triggering factors, severity, and pathological characteristics.<sup>5</sup> However, strong evidence supports the role of exposure to environmental dust in the pathophysiology of both mild–moderate and severe equine asthma.<sup>1</sup> Exposure to inhaled airborne agents, predominantly via stabling and provision of hay feeding, was identified as a risk factor for SA.<sup>6</sup> Controlled challenges and exposure studies have demonstrated an association between the severity of airway neutrophilic response in SA horses and exposure to  $\beta$ -glucan (marker of mould exposure), or with the concentration of mould spores.<sup>7,8</sup> In MA,  $\beta$ -glucan contents of respirable dust have been associated with increased proportions of mast cells in bronchoalveolar lavage fluid (BALF).<sup>9</sup> Moreover, a recent publication showed that horses with fungal elements detected in the tracheal wash (TW) cytology were twice as likely to have MA than horses with no fungi.<sup>10</sup> However, a retrospective study on 886 BALF samples showed that cytological evidence of fungal elements in BALF was not a risk factor for respiratory inflammation.<sup>11</sup>

For clinical purposes, questions remain about the most relevant sampling site and methodology for detection of fungal elements in equine airways. Laboratory analytical methods for fungal detection include either cytology or fungal culture of respiratory tract fluids. Poor agreement between those two techniques has previously been reported.<sup>10,12</sup> In TW samples, fungal detection is common, occurring in 55%–82% of samples depending on methodology,<sup>10,12</sup> rendering questionable the clinical significance of the presence of fungi in TW. Fungal detection in BALF is less common (estimated at 37% in a recent study<sup>12</sup>), with a poor agreement between the two sampling sites.<sup>12</sup> One rationale for performing fungal detection on TW samples is that this sample reflects more accurately the overall exposure of the equine airways to these particles.<sup>10</sup> However, as equine asthma is a disease of the lower respiratory tract,<sup>1</sup> investigating fungal detection in BALF may be a more accurate sampling site regarding the association of mould and development of equine asthma.

With the hypothesis that exposure to fungal elements are a risk factor for equine asthma, the aim of the study was to investigate different combinations of analytical methods/sampling sites in relation to the clinical status. The objectives were to: (1) determine the prevalence of fungal elements in different respiratory samples (TW or BALF) and methods (cytology or fungal culture), (2) compare the prevalence of fungal elements between groups (control, MA and SA), and (3) determine any eventual association between fungal detection and clinical status.

## 2 | MATERIALS AND METHODS

### 2.1 | Study population

The study included convenience sampling on 90 French Standardbred racehorses in active training, between October 2020 and January 2023; and voluntary recruitment of 84 client-owned horses referred to the hospital between February 2021 and May 2023 for decreased performance and/or respiratory disease. Minimal sample size was estimated at 92 horses (Epi InfoTM, Cohort and cross-sectional study statistical calculator) in order to be able to detect a significant odds ratio of 3.5. Data concerning housing conditions (indoor/outdoor), bedding (straw, wood shavings), hay forage (dry/soaked/steamed), activity (training/resting), 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. Horses that had received corticosteroids or antimicrobials within the 2 weeks before examination were not included in the study. Horses with signs of systemic illness, other causes of decreased performance or other respiratory pathologies than equine asthma were excluded.

Horses were sampled at least 24 h after the last training or race. Each horse underwent clinical examination by an European College of Equine Internal Medicine (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 haematology, and additional biochemical analysis when judged clinically necessary.

### 2.2 | Respiratory sampling

Horses were either sampled in their usual environment (at their stables) or at the hospital after a short (<2 h) transportation. Horses that were not racing or competing within 10 days following sampling were sedated with detomidine (10  $\mu$ g/kg IV, Sedomidine, Audevard), with the addition of butorphanol (10–20  $\mu$ g/kg IV, Torphadine, Dechra) when needed. A nose twitch was used when necessary. For horses racing or competing within 4–10 days following investigation, the procedures were achieved with a nose twitch only.

#### 2.2.1 | Tracheal wash

A 120 cm long videoendoscope (AOHUA, Medimage off-site; OLYMPUS in-house) was passed through one of the nostrils into the pharynx. The endoscope was then inserted into the proximal trachea, and the presence of blood in the trachea and the mainstem bronchi was graded from 0 to 4 based on a reported scoring system (tracheal blood score, TBS, Table S1).<sup>13</sup> Tracheal mucus was also scored

according to a previously published scale from 0 to 5 (Table S1).<sup>14</sup> A single-use double-lumen sterile catheter (Mila International Inc.) was passed through the canal of the endoscope, and 40 mL of warmed (37°C) sterile saline solution (0.9% NaCl) was infused and retrieved. The aspirated fluid was divided into a sterile dry tube for microbiology (bacteriology and mycology), and a tube containing EDTA for cytological analysis.

### 2.2.2 | Bronchoalveolar lavage

The BAL was performed using a flexible 320 cm long videoendoscope (AOHUA, Medimage off-site; OLYMPUS in-house). After visualisation of the carina, the endoscope was inserted into the 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 systematically discarded. At the end of lavage of the first lung side, the endoscope instrument channel was cleaned with a 60 mL bolus of sterile isotonic saline; then the endoscope was moved back to the carina and introduced in the contralateral lung and lavage procedure was repeated.

The volume of liquid collected and macroscopic assessment were recorded. A proportional volume of BALF from each lung was pooled in a jar to obtain a pooled BALF.

Endoscopes were sterilised between each horse by flushing the channels and soaking the endoscopes in disinfectant (Hexanios G + R, Anios Laboratories) as per manufacturer's recommendations. Left, right and pooled samples were then placed in a tube containing EDTA for cytology, and another aliquot of pooled samples was placed in a sterile tube for mycology. All samples were kept chilled until handled by the laboratory, and all analyses were performed within 24 h of sampling.

### 2.2.3 | Cytology

Three hundred microlitres of fluid from TW and BALF (left, right and pooled) were cytocentrifuged (80g, 10 min) and stained with May-Grünwald-Giemsa (MGG). A differential cell count was performed on 400 cells and the number of each cell type was recorded as a percentage of total nucleated cells, excluding epithelial cells. The presence of fungal particles (spores and hyphae, extracellular or phagocytosed) and bacteria was documented by assessment of the entire slide.

### 2.2.4 | Microbiology

Bacteriological examinations were performed as previously described.<sup>15,16</sup> Briefly, dilution series were done from liquid medium on agar plates, which were incubated for at least 24 h (Table S2), and cultures were considered positive when bacterial concentrations

reached 10<sup>4</sup> CFU/mL or more. The colonies were characterised by Gram staining, catalase, oxidase, glucose fermentation, and motility tests. Identification to species level was done by API system (Biomerieux).

Mycology testing was performed by direct isolation on selective media (Table S3) as previously described.<sup>12</sup> Briefly, 1 mL of the 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 staining with 0.4% Tryptan blue. 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 (2 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.

### 2.2.5 | Molecular biology

All qPCR have previously been validated in respiratory samples, based on the NF-U-47600-2 AFNOR norm.<sup>17</sup> Detection of the following viruses was performed in TW: equine herpesvirus (EHV) -1, -2, -4 and -5; equine rhinitis virus A (ERAV) and B (ERBV); equine adenovirus (EAdV) -1 and -2; and equine influenza virus (EIV). Herpesvirus-2, EHV-5 and ERBV were also assessed in the pooled BALF. Ten microlitres of TW and 25 mL of BALF were centrifuged (800g; 20 min; 4°C), and pellets were re-suspended with 1 mL of supernatant. Extraction of viral nucleic acids and virus specific qPCR were performed using previously reported assays.<sup>18</sup> Briefly, extraction of DNA/RNA was made from 140 µL of each sample, using the QIAamp<sup>®</sup> RNA viral Mini Kit (Qiagen) according to the manufacturer's instructions. Concentrated DNA/RNA was then stored at -80°C until used. Virus-specific qPCR were performed in a total volume of 25 µL, and included a negative control (distilled water) and serial dilutions of synthetic DNA/RNA (respectively plasmids and transcripts of plasmids for each targeted region). Amplification of the synthetic sequence 'myIC' was also performed, to confirm the absence of PCR inhibitors in the nucleic acid extract.

## 2.3 | Case definition

Based on the previous consensus statement,<sup>1</sup> a diagnosis of mild-moderate asthma (MA) was made based on the presence of clinical signs (poor performance (i.e., not performing as expected whatever level of activity), chronic occasional coughing (at least 4 weeks in duration), or both), evidence of airway inflammation in BALF cytology,

presence of significant amount of tracheal mucus detected by endoscopy (grade >2/5), and the exclusion of severe asthma (SA) based on history and clinical signs. For BALF cytology, the 'less restrictive' cut-off was used: neutrophils >10%, mast cells >5% and eosinophils >5% in individual (left and right) BALF,<sup>1,19</sup> and neutrophils >9%, mast cells >3% and eosinophils >3% in pooled BALF.<sup>20</sup> A diagnosis of SA was made based on history, presence of increased respiratory effort at rest, and moderate to severe neutrophilia in BALF (>25%) in horses of middle to advanced age (>7 years old).<sup>1</sup> Horses without coughing, nasal discharge, or increased respiratory effort, with normal BALF cytology from both lungs, and with a negative bacterial culture from TW were classified as controls (CTL). Microscopic detection was classified as positive when at least one fungal element (spore, hyphae) was observed on the slide (TW, individual or pooled BALF). Fungal culture was classified as positive when one or more colonies were observed (and identified) in a sample (TW or pooled BALF).

## 2.4 | Data analysis

Statistical analyses were performed using GraphPad Prism 10.3.0 software. A Chi-square test (or a Fisher's exact test when necessary) was performed to compare the final prevalences among groups according to each sample modality (Figure S1). Univariable associations between fungal detection and qualitative variables (extrinsic factors such as housing (pasture, box with straw or shavings) or feeding type (dry, soaked or steamed hay), clinical examination (clinical signs, tracheal mucus score, tracheal blood score), clinical status (control, mild-moderate or severe asthma), Table S1) were evaluated with a Chi-square test, as well as odds ratio, sensitivity, specificity and predictive values (Figure S1). Specificity, sensitivity and predictive values were arbitrarily described as poor when  $\leq 0.20$ , fair if  $0.21 \leq \text{value} \leq 0.40$ , moderate if  $0.41 \leq \text{value} \leq 0.60$ , substantial if  $0.61 \leq \text{value} \leq 0.80$  and good if value exceeds 0.80. Association between fungal detection and quantitative variables (neutrophil proportions) was determined using Cochran-Mantel-Haenszel test. Multivariable logistic regressions were performed, using a forward stepwise approach, by testing all variables with a previous univariable  $p$  value <0.1. Values of  $p < 0.05$  were considered statistically significant.

## 3 | RESULTS

### 3.1 | Study population

Among the 174 horses sampled, 3 horses were excluded because samples were uninterpretable on receipt at the laboratory, and 16 were excluded from the study because of respiratory issues unrelated to equine asthma: 10 with infectious pneumonia, 5 with exercise-induced pulmonary haemorrhage (EIPH) and 1 with interstitial pneumonia. Overall, 155 horses were included. The population was composed of 87 Standardbred racehorses (56.1%), 20 sport horses (12.9%), and 48 leisure horses (31%). Horses were aged from 2 to 25 years (median 6 years),

with a total of 38 entire males (24%), 60 geldings (39%) and 57 females (37%). Horses' sampling were performed at their stables for 91 horses and 64 horses were sampled at the hospital.

Of these, 85 horses were diagnosed with MA, 35 horses were SA and 35 horses were considered controls (CTL). Among the MA group, 29.4% (25/85) exhibited mast cell inflammation only, 2.4% (2/85) eosinophilic inflammation only, 43.5% (37/85) neutrophilic inflammation only, 11.8% (10/85) increased tracheal mucus score only and 12.9% (11/85) had a mixed pattern among the four inclusion criteria. All horses had a TBS of 0. Horses' characteristics across clinical status are described in Table S4.

### 3.2 | Bacterial and viral detection on airway samples

Bacteria were observed in the TW of 11 horses. *Streptococcus equi* subsp. *zooepidemicus* ( $2.0 \times 10^4$ – $1.2 \times 10^7$  CFU/mL) was identified in 6 horses, 4 horses with MA and 2 horses with SA. *Actinobacillus equuli* subsps. *haemolyticus* ( $1.0 \times 10^4$ – $2.0 \times 10^6$  CFU/mL) was identified in 5 samples, 3 horses with MA and 2 horses with SA. *Mycoplasma* spp. was detected in the TW of 34.3% (57/155) of all horses, with 31.6% (12/35) of CTL horses, 38.8% (33/85) of MA horses and 28.6% (12/35) of SA horses, respectively.

Herpesvirus-4 was detected in TW of 2 horses (1.3%), both were in MA group. Herpesvirus-2 and EHV-5 were detected in TW of 30.3% (47/155) and 38.1% (59/155) of all horses, respectively. Herpesvirus-2 was identified in 37.2% (13/35) of CTL horses, 28.2% (24/85) of MA horses and 34.3% (10/35) of SA horses. On the other hand, EHV-5 was identified in 40% (14/35) of CTL horses, 42.4% (36/85) of MA horses and 25.7% (9/35) of SA horses. Equine rhinitis virus B was detected in TW of 38.6% (59/153) of horses, with 42.4% (14/33) of CTL horses, 38.8% (33/85) of MA horses and 34.3% (12/35) of SA horses. Herpesvirus-1, ERAV, EAAdV-1 and -2 and EIV were not detected in any TW sample. In BALF, EHV-2 was identified in 8.4% (13/154) of horses, with 13.1% (11/84) in the MA group and 5.7% (2/35) in the SA group. Equine herpesvirus-5 was identified in 7.1% (11/154) of all samples, with 2.9% (1/35) in the CTL group, 9.5% (8/84) in the MA group and 5.7% (2/35) in the SA group. Equine rhinitis virus B was not detected in any BALF sample.

### 3.3 | Fungal detection in TW

Overall, a positive TW microscopic detection was obtained in 75.5% (117/155) of horses (Table 1). Proportion of samples positive for fungi on microscopic examination was significantly lower in the SA group than in CTL ( $p = 0.007$ ) and MA ( $p < 0.001$ ) groups (Table 1; Figure 1A). Similarly, the proportion of fungi phagocytosed by macrophages was significantly lower in the SA group than the CTL ( $p = 0.02$ ) and MA ( $p = 0.01$ ) groups. The proportion of samples with spores or hyphae (phagocytosed or not) was not significantly different between groups.

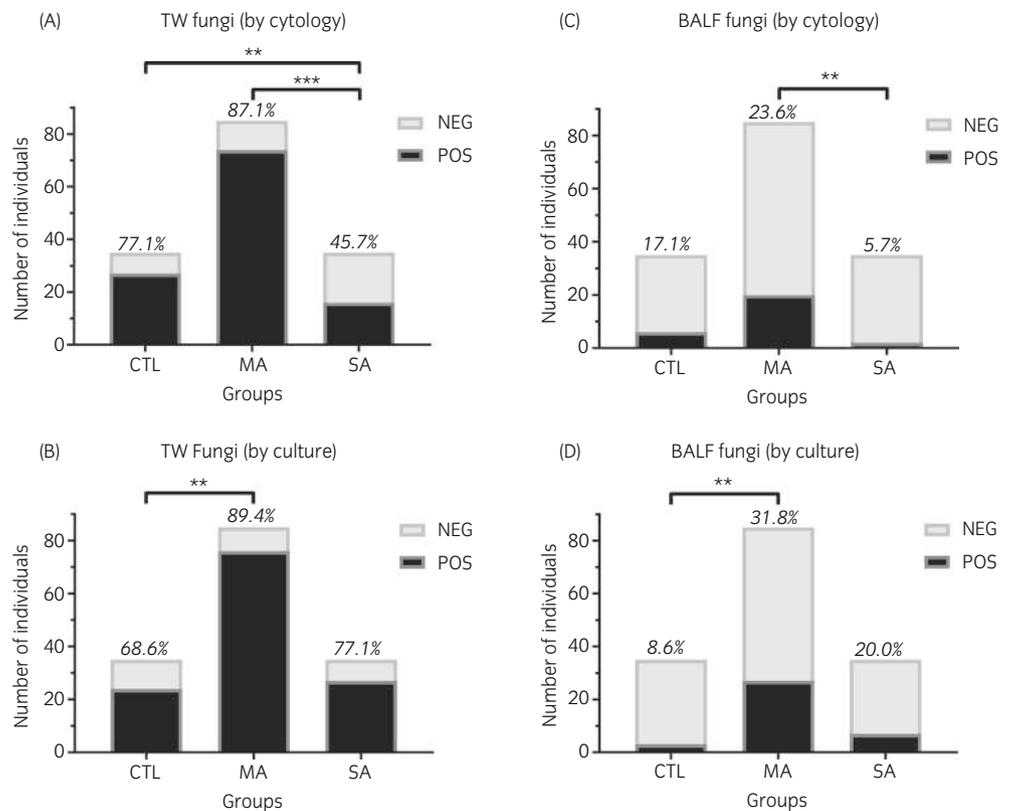
**TABLE 1** Prevalence of positive fungal microscopic detection and phagocytosis of fungal elements in tracheal wash according to clinical status.

	Fungal elements		Spores		Hyphae	
	Overall prevalence	Phagocytosed	Proportion among total	Phagocytosed	Proportion among total	Phagocytosed
General population	75.5% (117/155)	66.7% (78/117)	87.2% (102/117)	71.6% (73/102)	74.4% (87/117)	60.9% (53/87)
CTL	77.1% <sup>a</sup> (27/35)	74.1% <sup>a</sup> (20/27)	92.6% (25/27)	76.0% (19/25)	81.5% (22/27)	63.7% (14/22)
MA	87.1% <sup>a</sup> (74/85)	70.3% <sup>a</sup> (52/74)	87.8% (65/74)	73.8% (48/65)	73.0% (54/74)	64.8% (35/54)
SA	45.7% <sup>b</sup> (16/35)	37.5% <sup>b</sup> (6/16)	75% (12/16)	50.0% (6/12)	68.8% (11/16)	36.4% (4/11)

Note: Different letters correspond to significant differences between groups ( $p < 0.05$ ).

Abbreviations: CTL, control group; MA, mild-moderate asthma group; SA, severe asthma group.

**FIGURE 1** Proportions of positive fungal detection depending on clinical status and sampling site. (A) TW by cytology, (B) TW by culture, (C) BALF by cytology and (D) BALF by culture. TW, tracheal wash; BALF, bronchoalveolar lavage fluid; CTL, control group; MA, mild-moderate asthma group; SA, severe asthma group.



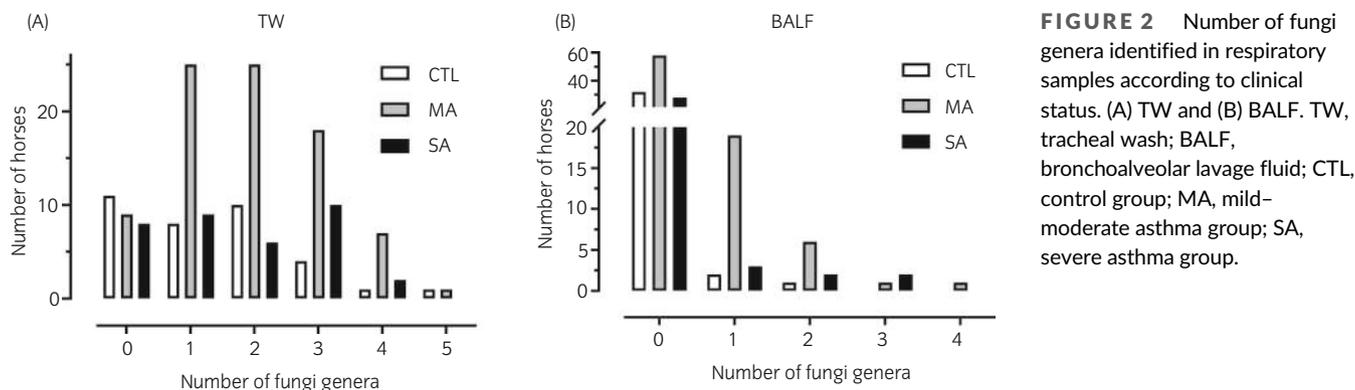
Overall, a positive TW mycology was obtained in 81.9% (127/155) of all horses. Proportion of samples positive for fungi by culture was significantly higher in the MA group than the CTL group ( $p = 0.005$ ) (Figure 1B). In the TW, 17 fungal *genera* were identified, with the most commonly isolated *genera* being *Aspergillus* spp. (80/127, 63%), *Penicillium* sp. (66/127, 52%), and *Chrysosporium* sp. (45/127, 35.4%), alone or in combination with other fungi (Table S5). Culture allowed identification of 7 species of *Aspergillus* spp., alone or in combination with other species: *A. nidulans* ( $n = 35$ ), *A. fumigatus* ( $n = 29$ ), *A. niger* ( $n = 15$ ), *A. glaucus* ( $n = 14$ ), *A. terreus* ( $n = 6$ ), *A. versicolor* ( $n = 6$ ) and *A. flavus* ( $n = 2$ ).

The number of fungal *genera* concomitantly identified according to samples and group is available in Figure 2. *Aspergillus* spp. was identified in 54.2% (13/24) of CTL horses, 68.4% (52/76) of

MA horses and in 55.6% (15/27) of SA horses, with no significant difference between groups. Proportions positive for *Penicillium* sp. were not significantly different between groups; this genus was found in 66.7% (16/24) of CTL horses, in 47.4% (36/76) of MA horses and in 51.9% (14/27) of SA horses. Similarly, proportions of *Chrysosporium* sp. did not differ significantly between groups, with 33.3% (8/24) in CTL horses, 36.8% (28/76) in MA horses and 33.3% (9/27) in SA horses.

### 3.4 | Fungal detection in BALF

Overall, a positive BALF microscopic detection was obtained in 18.1% (28/155) of all horses (Table 2). The proportion of positive



**FIGURE 2** Number of fungi genera identified in respiratory samples according to clinical status. (A) TW and (B) BALF. TW, tracheal wash; BALF, bronchoalveolar lavage fluid; CTL, control group; MA, mild-moderate asthma group; SA, severe asthma group.

**TABLE 2** Prevalence of positive fungal microscopic detection and phagocytosis of fungal elements in bronchoalveolar lavage fluid according to clinical status.

	Fungal elements		Spores		Hyphae	
	Overall prevalence	Phagocytosed	Proportion among total	Phagocytosed	Proportion among total	Phagocytosed
General population	18.1% (28/155)	89.3% (25/28)	100.0% (28/28)	89.3% (25/28)	50.0% (14/28)	92.9% (13/14)
CTL	17.1% <sup>a,b</sup> (6/35)	83.3% (5/6)	100.0% (6/6)	83.3% (5/6)	33.3% (2/6)	50.0% (1/2)
MA	23.6% <sup>b,c</sup> (20/85)	90.0% (18/20)	100.0% (20/20)	90% (18/20)	36.4% (10/20)	80.0% (8/10)
SA	5.7% <sup>a</sup> (2/35)	100.0% (2/2)	100.0% (2/2)	100.0% (2/2)	100.0% (2/2)	100.0% (2/2)

Note: Different letters or different symbols correspond to significant differences between groups ( $p < 0.05$ ).

Abbreviations: CTL, control group; MA, moderate asthma group; SA, severe asthma group.

samples for fungi on microscopic examination was significantly lower in the SA group than the MA group ( $p = 0.02$ ) but not the CTL group (Table 2; Figure 1C). No significant difference was found between CTL and MA groups. Proportions of fungi phagocytosed by macrophages and proportions of spores or hyphae (phagocytosed or not) were not significantly different between groups.

Overall, a positive BALF mycology was obtained in 23.9% (37/155) of horses. The proportion of positive samples for fungi by culture was significantly higher in the MA group than the CTL group ( $p = 0.008$ ) (Figure 1D). In BALF, 8 fungal genera were identified (Table S6), with the most commonly isolated genera being *Aspergillus* spp. (56.8%, 21/37), *Aureobasidium* sp. (27%, 10/37) and *Penicillium* sp. (21.6%, 8/37). *Aureobasidium* sp. was however not detected in any TW sample. Culture allowed identification of 6 species of *Aspergillus* spp., alone or in combination with other species: *A. fumigatus* ( $n = 8$ ), *A. nidulans* ( $n = 7$ ), *A. glaucus* ( $n = 7$ ), *A. niger* ( $n = 3$ ), *A. versicolor* ( $n = 2$ ) and *A. terreus* ( $n = 1$ ).

*Aspergillus* spp. was isolated in 100% (3/3) of CTL horses, 55.6% (15/27) of MA horses and in 42.9% (3/7) of SA horses, with no significant difference between groups. Proportions of *Aureobasidium* sp. was not significantly different between groups; this genus was found in 33.3% (1/3) of CTL horses, in 33.3% (9/27) of MA horses and in 0% (0/7) of SA horses. Similarly, proportions of *Penicillium* sp. did not differ significantly between groups, with 0%

(0/3) in CTL horses, 14.8% (4/27) in MA horses and 57.1% (4/7) in SA horses. Horses with fungi identified in both TW and BALF are detailed in Table S7.

### 3.5 | Univariable association between fungal detection and extrinsic factors

The likelihood of finding fungal elements in TW and BALF by culture and microscopic detection was not significantly associated with either housing (pasture, straw or shavings) or feeding (dry, soaked or steamed hay). No significant associations were found between fungal detection in TW or BALF by culture or cytology and identification of bacteria in TW, identification of *Mycoplasma* spp. in TW or identification of virus in TW. Horses where EHV-2 was detected in BALF higher odds of exhibiting a positive fungal culture in BALF (OR = 2.42, 95% CI 1.03–5.35; Table 3), rather than negative fungal culture.

### 3.6 | Univariable associations between fungal detection and clinical examination

The prevalence of fungal elements either in TW or BALF by culture was not significantly associated with clinical signs (cough, nasal discharge,

**TABLE 3** Univariable association between fungal detection and extrinsic factors/clinical examination.

Variable	Fungal detection	Odds ratio	Lower 95% CI	Upper 95% CI	P-value
Positive EHV-2 detection in BALF	Negative fungal detection by culture in BALF	Ref	–	–	–
	Positive fungal detection by culture in BALF	2.42	1.03	5.35	0.03
Horses displaying cough	Negative fungal detection by cytology in TW	Ref	–	–	–
	Positive fungal detection by cytology in TW	0.33	0.16	0.68	0.003
Horses displaying cough	Negative fungal detection by cytology in BALF	Ref	–	–	–
	Positive fungal detection by cytology in BALF	0.27	0.11	0.73	0.008
Tracheal mucus score >2	Negative fungal detection by cytology in TW	Ref	–	–	–
	Positive fungal detection by cytology in TW	0.19	0.09	0.41	< 0.001
Tracheal mucus score >2	Negative fungal detection by culture in BALF	Ref	–	–	–
	Positive fungal detection by culture in BALF	2.26	1.03	4.85	0.03
Increased proportion of neutrophils (>10%) in BALF	Negative fungal detection by culture in BALF	Ref	–	–	–
	Positive fungal detection by culture in BALF	2.23	1.06	4.93	0.04

**TABLE 4** Univariable association between fungal detection and clinical status.

Variable	Clinical status	Odds ratio (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Positive cytology in TW	CTL	Ref.	–	–	–	–
	SA	0.25 (0.09–0.70)	0.70 (0.52–0.84)	0.63 (0.48–0.76)	0.54 (0.38–0.70)	0.77 (0.61–0.88)
Positive cytology in TW	SA	Ref.	–	–	–	–
	MA	7.99 (3.14–19.74)	0.63 (0.46–0.78)	0.82 (0.73–0.89)	0.54 (0.38–0.70)	0.87 (0.78–0.93)
Positive mycology in TW	CTL	Ref.	–	–	–	–
	MA	3.87 (1.37–10.08)	0.55 (0.34–0.74)	0.76 (0.67–0.83)	0.31 (0.19–0.48)	0.89 (0.81–0.94)
Positive cytology in BALF	SA	Ref.	–	–	–	–
	MA	5.08 (1.30–22.86)	0.34 (0.25–0.44)	0.91 (0.72–0.98)	0.94 (0.81–0.99)	0.24 (0.16–0.24)
Positive mycology in BALF	CTL	Ref.	–	–	–	–
	MA	4.97 (1.49–16.38)	0.36 (0.26–0.46)	0.90 (0.74–0.97)	0.91 (0.78–0.97)	0.32 (0.23–0.42)

Note: Variables were displayed only when  $p < 0.05$ .

Abbreviations: CI, confidence interval; CTL, control group; MA, mild–moderate asthma group; NPV, negative predictive value; PPV, positive predictive value; SA, severe asthma group.

dyspnea, wheezes or crackles). Horses with cough were significantly less likely to exhibit fungi by microscopic detection in TW and BALF (Table 3). Horses with a tracheal mucus score >2 were significantly less likely to exhibit fungi by microscopic detection in TW. Horses with a tracheal mucus score >2 had higher odds of having a positive fungal culture in BALF (OR = 2.26, 95% CI 1.03–4.85; Table 3) rather than negative fungal culture. Horses with an increased proportion of neutrophils in BALF had higher odds of exhibiting fungi by culture in BALF (OR = 2.23, 95% CI 1.06–4.93; Table 3) rather than negative fungal culture. The magnitude of inflammatory neutrophilic reaction, when present (>10%), was however not significantly associated with fungal detection in BALF (Table S8). No association was found between fungal detection by cytology in BALF and the proportion of neutrophils, nor between fungal detection (by culture or cytology) in BALF and the proportion of mast cells.

### 3.7 | Associations between fungal detection and clinical status

No significant association was observed between clinical status (MA vs. CTL) of horses and positive microscopic detection in TW. Horses with SA were significantly less likely to exhibit a positive microscopic detection in TW compared with CTL (OR = 0.25, 95% CI 0.09–0.70), with substantial sensitivity and specificity, moderate positive predictive value (PPV) and substantial negative predictive value (NPV) (Table 4; Figure 1A). Horses with MA had higher odds of positive microscopic detection in TW compared with SA horses (OR = 7.99, 95% CI 3.14–19.74), with substantial sensitivity, good specificity, and moderate PPV and good NPV (Table 4; Figure 1A).

**TABLE 5** Multivariable logistic regression model for diagnosis of mild–moderate asthma.

Variable	$\beta$	SE ( $\beta$ )	OR	95% CI (OR)	$p$
TW mycology					
Negative	–	–	Ref.	–	–
Positive	1.24	0.73	3.44	0.84–15.82	0.09
BALF mycology					
Negative	–	–	Ref.	–	–
Positive	<b>1.65</b>	<b>0.70</b>	<b>5.21</b>	<b>1.49–24.89</b>	<b>0.02</b>
Environment/bedding					
Pasture	–	–	Ref.	–	–
Straw	<b>–1.17</b>	<b>0.52</b>	<b>0.31</b>	<b>0.11–0.85</b>	<b>0.03</b>
Wood shavings	–1.78	1.14	0.17	0.02–1.58	0.12
Feeding (hay)					
Dry	–	–	Ref.	–	–
Soaked	1.97	1.32	7.15	0.71–180.0	0.14
Steamed	0.69	1.09	2.00	0.29–25.09	0.53

Note: Variables were displayed in bold when  $p < 0.05$ .

Abbreviations: OR, odds ratio; SE ( $\beta$ ), standard error;  $\beta$ , model coefficient.

No significant association was observed between status (SA vs. either CTL or MA) of horses and positive mycology culture in TW. Horses with MA had higher odds of positive mycology in TW compared with CTL horses (OR = 3.87, 95% CI 1.37–10.08), with moderate sensitivity, a substantial specificity, and fair PPV and good NPV (Table 4; Figure 1B).

No significant association was observed between status (MA vs. CTL or SA vs. CTL) of horses and positive microscopic detection in BALF. Horses with MA had higher odds of positive microscopic detection in BALF compared with SA horses (OR = 5.08, 95% CI 1.30–22.86), with fair sensitivity, good specificity, and good PPV and a poor NPV (Table 4; Figure 1C).

Horses with MA had higher odds of positive mycology in BALF compared with CTL horses (OR = 4.97, 95% CI 1.49–16.38), with poor sensitivity, good specificity, and good PPV and poor NPV (Table 4; Figure 1D).

Feeding, bedding, TW mycology and BALF mycology exhibited  $p < 0.1$  in univariable regression and were then included into multivariable model. There was significantly increased odds of MA diagnosis also observed in association with a positive BALF mycology, while significantly decreased odds was observed with straw bedding (Table 5). Similarly, significantly increased odds of SA diagnosis were observed in association with both soaking and steaming hay for feeding, while significant decreased odds was observed with straw as stall bedding (Table S9).

## 4 | DISCUSSION

Our study demonstrated that fungal elements were commonly present in the trachea, with a positive TW mycology obtained in 81.9%

of samples and fungal elements present upon cytological examination of TW in 75.5% of samples. In contrast, fungal detection was scarce in the BALF, with fungal elements found upon cytological examination in 18.1% of samples and a positive BALF mycology culture obtained in 23.9% of samples. The results from this study partially supported our hypothesis that exposure to fungal elements was a risk factor for asthma, since only horses with MA had almost 5-fold higher odds of positive mycology in BALF compared with CTL horses. Furthermore, horses with an increased score of tracheal mucus were more likely to exhibit positive fungal culture in BALF, as well as horses with an increased proportion of neutrophils in BALF.

### 4.1 | Analytical methods of fungal detection

In order to refine clinical interpretation, potential cytological markers have been investigated in our study, such as identification of type of fungal elements (spores or hyphae) and whether they were phagocytosed or not. As spores represent a dormancy form and hyphae a growing form of fungi, the latter have previously been evaluated in the context of MA.<sup>10</sup> Our results showed that identification of growth as well as phagocytosis of fungal elements is not significantly correlated with either clinical signs, inflammation or diagnosis of equine asthma; thus precluding the use of these as biological markers of occurrence or severity of asthma in horses.

A high prevalence of fungal detection by cytology in TW of non-asthmatic horses has been reported previously,<sup>10,21,22</sup> and also confirmed in the current study. Moreover, as there is no significant difference between the prevalence of fungi by microscopic detection in BALF of CTL and MA horses, this result drives to the conclusion that this method is not relevant for the investigation of fungi elements in athletic horses. Indeed, horses with a positive microscopic detection in TW or BALF were less likely to exhibit cough, because of the very large proportions of fungi detected by this method among control horses with no clinical signs. However, the results of the current study did identify a significant association between fungal detection by microscopic detection in BALF and SA. Severe asthmatic horses were indeed prone to exhibit fewer fungal elements compared with moderate asthmatics. This finding concurs with a previous study in which intracellular fungal-like particles were less commonly observed in the macrophages of severe asthmatic horses exposed to hay, compared with healthy horses.<sup>23</sup> The authors suggested that fungal particles were trapped more proximally in the respiratory tract, due to increased mucus and bronchoconstriction. An alternative hypothesis would be a decreased phagocytic capacity of macrophages of horses with SA, as previously demonstrated *ex vivo*.<sup>24</sup>

### 4.2 | Prevalence of fungal detection

A several number of fungal *genera* were identified in this study, with *Aspergillus* spp. and *Penicillium* being the most commonly found in respiratory fluids. While similar *genera* have been reported in previous

studies,<sup>10,25</sup> up to 5 different *genera* or up to 5 different species of *Aspergillus* spp. could be concomitantly identified in the airways of a single horse. It has previously been documented that *Penicillium*, *Aspergillus* spp., and *Mucoraceae genera* were airborne, ubiquitous and frequently found in the air of stables<sup>26</sup> while *Aspergillus* spp. is the most commonly isolated fungi in hay.<sup>12,27</sup> Detection of these fungal elements in the airways may simply reflect the environment in which the horse was located before and during sample collection.<sup>10,21,28,29</sup> Indeed, the authors of a recent publication identified the fungal microbiota present along the upper and lower respiratory tract of healthy horses, and showed that fungal microbiota of the pharynx was similar to that of the trachea in healthy horses, indicating that the pharynx is potentially a major source of microbes that translocate to the lower respiratory tract.<sup>25</sup> Even though an association between fungal content in hay samples and fungal elements in the airways of horses has not been identified,<sup>12</sup> other sources of contamination in horses' stables remain to be investigated.

In the current study, the *genus Aureobasidium* sp. was found in BALF only (and not in TW) of 10 horses (9 MA, 1 CTL). In horses, few anecdotal studies reported the detection of *Aureobasidium* sp. in fungal microbiota of the conjunctiva of clinically healthy horses,<sup>30</sup> in paranasal sinus of healthy and diseased horses<sup>31</sup> and through ITS2 gene sequencing (mycobiota) in BALF of horses.<sup>32</sup> However, the existence of an immune-mediated hypersensitivity reaction leading to interstitial pneumonitis due to *Aureobasidium pullulans* has been reported in the human literature.<sup>33</sup> Further studies are required to confirm the possible pathogenicity of *Aureobasidium* sp. and determine whether specific *genera* would present more risk than others for the development of equine asthma. Possibly, some other factors (such as relative size, shape and density of the spores) may also play a role in the possible pathogenicity of the fungal elements.

### 4.3 | Association between fungal detection and other infectious agents

To our knowledge, this is the first study to record detection of multiple types of infectious agents (bacterial, viral, fungal and *Mycoplasma* spp.) in both TW and BALF, and to examine their associations with equine asthma. Isolation of bacteria in TW has previously been associated with lower airway inflammation.<sup>34–37</sup> However, due to a very low prevalence of positive bacterial samples in the present study, it was not possible to confirm this association with moderate or severe equine asthma. Alternatively, frequent co-detection of fungal elements and other infectious agents such as viruses or mycoplasmas has been highlighted in this study. *Mycoplasma* spp. were recently found to be detected in 19.4% of TW samples from horses with respiratory diseases,<sup>38</sup> although their detection in TW was not significantly different between groups (CTL, MA, SA) in the current study. The potential implication of mycoplasmas in equine asthma remains to be determined, especially by quantitative investigation as per other bacteria in TW.

Our results indicated a significant positive association between fungal detection by culture in BALF and detection of EHV-2 in BALF.

So far, the evidence of a potential role of respiratory viruses is controversial; some studies report an association between MA and genome detection by PCR for EHV-2, EHV-5 and ERBV,<sup>18,39</sup> while others do not.<sup>40,41</sup> There has been speculation that these viruses may act as immunosuppressive agents, predisposing to other infections,<sup>42</sup> or may be involved in persistent, chronic fatigue syndromes.<sup>43</sup> Larger multicentric studies would be required to determine the possible synergy between viruses, bacteria, fungi, particles etc. (interactome) in the pathophysiology of equine asthma.

### 4.4 | Association between fungal detection and inflammatory response

A significant positive association was found between fungal detection by culture in BALF and a high tracheal mucus score, as well as with a high percentage of neutrophils in BALF. When present (>10%) the magnitude of neutrophilic response was however not significantly associated with the prevalence of mould in BALF. Tracheal mucus accumulation and neutrophil proportions are evidence of airway inflammation, but without specific cause.<sup>5</sup>

Fungal exposure and its association with SA have frequently been documented,<sup>8,44,45</sup> but studies examining fungal association with MA are still scarce. One study found that horses with fungal elements observed in TW microscopic examination had twice the odds of having IAD than horses without fungi.<sup>10</sup> There is also little information to date concerning the association between antigenic triggers and specific MA phenotypes.<sup>46</sup> Neutrophilic airway inflammation in BALF of racing Thoroughbreds has been correlated with both respirable dust and endotoxin exposure, while mast cell inflammation in BALF has been associated with  $\beta$ -glucan exposure.<sup>9</sup> Interestingly, while both univariable and multivariable analyses demonstrated the association between fungal detection by culture and MA diagnosis, our results also tend to demonstrate the lack of such with non-neutrophilic forms (mast-cell and eosinophilic) of pulmonary inflammation. These elements suggest that fungal detection in the airways is not linked to a specific immune response.

## 5 | LIMITATIONS

Fungal detection in BALF was performed on pooled BALF only, and not on individual right and left BALF, which could have led to an overall lower prevalence of fungal detection by culture in BALF. In a previous study, while 100% of horses with 'positive' pooled BALF were also positive in left and/or right BALF, 14.6% of horses with 'negative' pooled BALF were positive in left and/or right BALF.<sup>12</sup>

Beyond detection of fungi in TW or BALF, quantification of the fungal load in the airways also needs to be further investigated. Cytological investigations of fungal elements were limited to qualitative dichotomy (presence or absence) only. Quantification of fungal-like intracellular particles by cytology or total fungal load quantification by qPCR assay (18S rRNA gene) have recently been proposed, but these

methods are yet to be validated.<sup>23</sup> Absolute quantification by successive dilutions (as performed for bacteria in TW for instance)<sup>16</sup> were laborious, unrepeatable (data not shown) and ultimately unsuccessful for fungal cultures. However, establishment of a 'pathological' cut-off, possibly through molecular biology, could however help in the future for differentiating true fungal infection from environmental contamination.

Similar to asthma of humans, asthma of horses is a multifactorial process, with a wide heterogeneity in terms of triggering factors suspected to be involved in the occurrence of the disease. While the current clinical study focused on fungal detection in the airways and disease status, potential confounding factors—such as bedding or forage—were found to significantly be involved as well. Indeed, as horses were recruited on a voluntary basis, some environmental alterations were already applied by the owners for several (mild or severe) asthmatic horses prior to sampling, and could not be controlled in the design of this clinical study. This method of recruitment may then have allowed for a selection bias. Apart from the environmental description of the cases, larger epidemiological studies are still required in order to further characterise the influence of airborne fungal exposure on (mild) asthma of horses.

Additionally, ambient airborne samples or component of horses' environment (bedding, food) were not collected. No association between fungal content in hay samples and fungal elements in the airways of horses was detected in a previous study.<sup>12</sup> It would however have been interesting to collect respirable and inhaled particulate samples in the horse's breathing zone, as previously described.<sup>47,48</sup> The relevance of such sampling, factually excluding horses referred to the hospital for consultation, is however limited to field studies only, most frequently focusing on MA rather than SA.

## 6 | CONCLUSION

Overall, the prevalence of fungal detection was found to be significantly higher in the airways of MA horses than SA and/or CTL horses. Due to the high proportion of positive fungal detection in TW of CTL horses, by either microscopic detection or culture, investigations based on such samples were found to be uninformative in a clinical context. On the other hand, fungal detection by culture (but not by cytology) in BALF has been identified as a significant risk factor for mild-moderate asthma in horses.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

**Pauline Barbazanges:** Formal analysis; visualization; writing – original draft; data curation; investigation; writing – review and editing; validation; methodology. **Anne Courouc :** Conceptualization; data curation; formal analysis; visualization; writing – original draft; methodology; investigation; supervision; project administration; writing – review and editing; validation; funding acquisition. **Gabin Le Digarcher:** Investigation; data curation. **Jacqueline M. Cardwell:** Conceptualization; supervision; writing – review and editing. **Elliott Schmitt:** Investigation; data curation. **Marie-Pierre Toquet:** Conceptualization; data curation; formal analysis; writing – original draft; investigation; methodology. **Louise C. Lemonnier:** Data curation; methodology; investigation; writing – original draft. **Eric A. Richard:** Conceptualization; data curation; formal analysis; visualization; writing – original draft; methodology; investigation; supervision; project administration; writing – review and editing; software; validation; funding acquisition.

## DATA INTEGRITY STATEMENT

Pauline Barbazanges, Anne Courouc  and Eric A. Richard had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of data analysis.

## ETHICAL ANIMAL RESEARCH

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

## INFORMED CONSENT

Owners gave consent for their animals' inclusion in the study.

## PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.14470>.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request: Open sharing exemption granted by editor for this clinical report.

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### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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