Determination of the mycobiome in the lower respiratory tract of horses with equine asthma

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Abstract

Fungal particles are important allergenic components involved in the development of equine asthma. The aim of this study was to evaluate the mycobiome composition of the lower respiratory tract in asthmatic horses using fungal culture, quantitative multiplex real-time PCR analysis (FungiMultiPlex) and Next-Generation Sequencing approach. Bronchoalveolar lavage fluid (BALF) samples obtained from 45 client-owned horses diagnosed with equine asthma were analysed by fungal culture (19 samples), FungiMultiPlex (34 samples), and Next-Generation Sequencing (14 samples). The fungal culture was positive in 11/19 (58%) cases, and FungiMultiPlex was positive in 19/34 (56%) cases. No fungal PCR product was detected by Next-Generation Sequencing analysis. Fungal culture and FungiMultiPlex methods were performed simultaneously on eight horses only. Association of results of these methods was calculated using Phi coefficient (φ= 0.333), and concordance between the methods was not confirmed (P = 0.420). The results of this study suggest that the fungal culture and quantitative multiplex real-time PCR might be considered diagnostically useful to assess the presence of fungi in BALF in a semiquantitative and quantitative manner, respectively. The Next-Generation Sequencing method seems to be less diagnostically suitable due to technical obstacles pertinent to both the low concentration of microbial agents in the rather diluted BALF samples, and, also, due to the relatively high environmental background contamination of the collected material. Based on our data, we advocate the use of the combination of quantitative multiplex real-time PCR and fungal culture in a routine clinical diagnostic setting.

Fungi, fungal culture, real-time PCR, Next-Generation Sequencing

Equine asthma (EA) represents a common cause of poor performance in sport horses. Severe forms of EA lead to exercise intolerance and clinical signs of respiratory distress whilst at rest. The main cause of asthma includes high concentrations of particles in the air in closed stables, i.e. dust, endotoxin, fungi and their spores, ultra-small particles as well as irritating gases (Beeler-Marfisi et al. 2010; Bond et al. 2020). Mild to moderate EA affects 68–80% of both pleasure horses and racehorses; the prevalence of severe EA is estimated to be 14–17% (Hotchkiss et al. 2007; Wasko et al. 2011; Ivester et al. 2018; Couetil et al. 2020).

The treatment of asthma consists of a combination of reducing environmental dust and administering drugs. The drugs used are mainly corticosteroids, mucolytics, and bronchodilators (Couëtil et al. 2016). Fungal lung populations have only been described in horses with mild to moderate asthma, but have not yet been described in horses with severe asthma (Dauvillier et al. 2019). The pathogenicity of fungi, i.e. their allergenic, toxic and infectious effects, has already been described in people with asthma, and targeted

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antifungal treatment has reduced clinical symptoms in up to a third in these patients (Rick et al. 2016). In horses with mild to moderate asthma, fungi were cultured in 55% of the cases. However, on cytological specimens from tracheal mucus, they were observed in 79% of the cases (Dauvillier et al. 2019). A study has been done by simultaneously determining the biomarkers galactomannan (GM) and (1,3) β-D-glucan (BDG) in serum samples from horses with equine asthma and the DNA of *Aspergillus*, galactomannan (GM), ferricrocin (Fc), triacetyfusarinin C (TafC) and gliotoxin (Gtx) in bronchoalveolar lavages (Dobiáš et al. 2023).

The aim of our study was to assess the composition of mycobiome in the lower respiratory tract in horses with EA by both classic and novel molecular laboratory methods - fungal culture, quantitative multiplex real-time PCR analysis (FungiMultiPlex) and Next-Generation Sequencing (NGS).

Materials and Methods

Horses

Forty-five patients diagnosed with EA admitted to the Equine Clinic of the University of Veterinary Sciences Brno during the period from November 2019 to July 2021 were enrolled in a prospective manner and the following indicators were recorded:

- Signalment and history: age, sex, breed, housing conditions (stable air quality; type of building; method of feeding - quantity and quality of hay, soaking/steaming, haylage feeding; bedding type - straw/sawdust; housing regime and time of stay in the stable/pasture), owners' complaints, and previous treatment;
- Haematological indices;
- Clinical parameters: nutritional status, consciousness, examination of the cardiovascular and respiratory system, findings on other organs systems.

Horses with EA were divided into two groups: mild to moderate asthma and severe asthma according to the 2016 ACVIM Consensus Statement (Couëtil et al. 2016).

Among the 45 horses diagnosed with EA, 25 were geldings, 18 mares, and 2 stallions. The mean age was 11 years (range 2–20 years). The majority were Warmbloods (19), followed by mix breeds (9), Thoroughbred (5), American Quarter Horse (3), Lusitano (2), pony (2), Paint Horse (1), Lipizzaner (1), Shagya Arab (1), and Kladruber (1). Mild to moderate EA was found in 23/45 horses, and severe EA in 22/45 horses.

Diagnostic procedures

Endoscopic examination (Storz, Vienna, Austria) of the respiratory tract was performed, and the amount and character of mucus in the trachea was graded for diagnostic purposes and the asthma classified according to Gerber et al. (2004).

Bronchoalveolar lavage fluid (BALF) was collected with a sterile collection kit (Kruuse, Langeskov, Denmark), using 300 ml sterile, body-warmed saline administered in 5 boluses. Cytological analysis of BALF was performed immediately after collection using a modified sedimentation technique (Day et al. 1995; Dauvillier et al. 2019). After the sedimentation, the BALF samples were stained with May-Grünwald Giemsa stain (Diapath, Martinengo, Italy). A sample of the last fraction of the BALF was taken into a sterile 20 ml syringe (Braun, Melsungen, Germany), from which 5 ml were immediately frozen and stored in a syringe at −80 °C. Fourteen random frozen samples were shipped to Tilia laboratories (Pchery, Czech Republic) for performing NGS analysis before the end of the study. The rest of the last fraction of the BALF sample (approx. 15 ml) was transferred to a Falcon tube (Biogen, Prague, Czech Republic) and sent within 24 h to the State Veterinary Institute in Hradec Králové (Czech Republic) for fungal culture.

Fungal culture

Fungi were cultured using Sabouraud's agar (Biolife Italiana, Milan, Italy), using two plates for one sample. Cultivation took place in a special incubator (Memmert, Schwabach, Germany) at 25 °C for at least 5 to 7 days. The identification was based on the assessment of colony morphology in the native preparation by microscopy (Olympus, Tokyo, Japan) and further by the growth rate of colonies on agar. Isolated cultures transferred to a slide were stained with lactophenol at the State Veterinary Institute Prague (Czech Republic).

Real-time PCR analysis (FungiMultiPlex)

The quantitative real-time PCR evaluation of a panel of mucosa-pathogenic fungi (FungiMultiPlex, Chambon, Czech Republic) was carried out according to the instructions of the manufacturer. The panel quantitatively detects the following microorganisms: *Candida albicans, C. krusei, C. glabrata, C. tropicalis, C. dubliniensis, C. inconspicua, C. lusitaniae, C. fabianii, C. parapsilosis, C. tropicalis, C. gulliermondii, Fusarium* spp.*, Cladosporium cladosporioides, Pneumocystis jiroveci, Rhizopus microsporus, R. oryzae, Mucor plumbeus, M. indicus, Cryptococcus* spp. (*Cryptococcus aureus, C. carnescens, C. flavescens, C. neoformans*),

Aspergillus fumigatus, Aspergillus spp. (*Aspergillus flavus, A. oryzae, A. nidulans, A. versicolor, A. ustus, A. cervinus, A. candidus, A. niger, A. terreus*).

Next-Generation Sequencing

Isolation of total DNA from frozen BALF samples disintegrated by SeptiFast Lys kit and MagNA Lyzer instrument ROCHE (Mannheim, Germany) was performed by DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

Next Generation Sequencing (NGS) was used for pan-detection of fungi (panF). Isolated DNA was subjected to end-point PCR amplification of the internal transcribed spacer (ITS) portion of 18S rDNA using primers PanF forward: ATTGGAGGGCAAGTCTGGTG and PanF reverse: CCGATCCCTAGTCGGCATAGTT. The NGS microbiome analysis was carried out as described in Načeradská et al (2021). Obtained raw data were end- and quality-trimmed and for the contig building only quality-filtered NGS reads were used. Only reads 200 bp and longer (up to 650 bp) were used for the read overlap (plus and minus strands) and contig building using Codon Code Aligner software (Codoncode Corporation, Centerville, Massachusetts, USA) was performed. Individual contigs were directly aligned to the genomic reference sequences depository located on the NCBI server (https://www.ncbi.nlm.nih.gov/).

Statistical analysis

Association between the results of the fungal culture and FungiMultiPlex method was calculated as Phi coefficient (known also as Mean square contingency coefficient). Significance was set at $P < 0.05$.

Results

Fourteen out of 45 samples were analysed by the NGS but no fungal PCR product was detected. Therefore, it was decided not to investigate the remaining samples for mycobiome by NGS.

Thirty-four BALF samples were analysed by quantitative real-time PCR FungiMultiPlex method. FungiMultiPlex was positive in 19 (56%) samples. *Aspergillus* spp. were found in 17 samples, *Aspergillus fumigatus* in 5 samples, *Pneumocystis jiroveci* in 2 samples, *Candida* spp. in 1 sample.

Nineteen BALF samples were examined by fungal cultivation. Fungal culture was positive in 11 (58%) samples. *Aspergillus niger* was cultivated in 6 samples, *Aspergillus fumigatus* in 5 cases, *Aspergillus parasiticus* in 5 cases, *Aspergillus flavus* and *Candida* spp. in 1 case.

Both the fungal cultivation and the real-time PCR were performed in eight samples. Results were slightly contradictory, as in six cases (67%) both methods yielded the same result (one double-negative and five double-positive samples), but in two cases the result was different (in one sample the fungal cultivation was negative, whereas multiplex real-time PCR positive, another one sample had opposite results - positive classical cultivation and negative real-time PCR). Phi coefficient of association was 0.333 and concordance of methods was not confirmed ($P = 0.420$). Detailed results of both methods performed simultaneously is shown in Table 1.

The most prevalent fungi found in the BALF samples, regardless of the method, were *Aspergillus* spp. in 23/45 (51%) samples, followed by *Candida* spp. in 3/45 (7%) samples, and *Pneumocystis* spp. in 2/45 (4%) samples.

Fungal cultivation revealed the presence of fungi in 4/8 (50%) horses with mild to moderate EA, and in 7/11 (64%) horses with severe EA. Fungi were detected by FungiMultiPlex in 9/19 (47%) horses with mild to moderate EA, and in 11/15 (73%) horses with severe EA.

Discussion

To the best of our knowledge, this is the first study where both the fungal cultivation and the molecular biological methods were used and compared to detect the mycotic organisms in BALF from horses with EA.

Fungal cultivation was positive in 58% of cases which is in accordance with previous studies on similar multi-breed groups of horses (Dauvillier et al. 2019). In racehorses,

Table 1. Results of fungal cultivation and FungiMultiPlex method performed simultaneously on bronchoalveolar lavage fluid samples obtained from eight client-owned horses diagnosed with equine asthma.

Sample No.	Breed	Age (years)	Sex	Asthma grade	Fungal cultivation	FungiMultiPlex
1	Quarter Horse	9	Stallion	Severe	*Aspergillus niger* (++++)	*Aspergillus* spp. 3×10^2/ml
2	Warmblood	13	Gelding	Mild to Moderate	*Aspergillus fumigatus* (+), *Aspergillus parasiticus* (+)	*Aspergillus* spp. 2×10^2/ml
3	Lipizzaner	11	Mare	Mild to Moderate	*Aspergillus fumigatus* (++), *Aspergillus niger* (+), *Aspergillus parasiticus* (+++)	*Aspergillus* spp. 3×10^2/ml
4	Mix breed	3	Gelding	Mild to Moderate	*Aspergillus niger* (++++)	Negative
5	Quarter Horse	13	Mare	Severe	*Aspergillus flavus* (+), *Aspergillus niger* (+)	*Aspergillus* spp. 1×10^3/ml, *Aspergillus fumigatus*, 2×10^2/ml, *Pneumocystis jiroveci* $\times10^2$/ml
6	Thoroughbred	14	Mare	Mild to Moderate	Negative	Negative
7	Thoroughbred	19	Gelding	Severe	*Aspergillus niger* (+)	*Aspergillus* spp. 2×10^2/ml
8	Shagya Arab	12	Gelding	Severe	Negative	*Aspergillus* spp. 3×10^2/ml

+, ++, +++, ++++ denote growth intensity of 1–10, 11–50, 51–300, > 300 colonies per plate, respectively

the positivity of BALF fungal cultivation was much lower (23–27%) (Lemonnier et al. 2022). The clinical significance of the finding is questionable, because prevalence of fungi in the BALF samples was similar for horses with and without EA (Dauvillier et al. 2019).

The NGS method using Ion Torrent, the method used in this study, enables detection of microorganisms at the species level (Načeradská et al. 2021). Another NGS technique used in other studies, Illumina sequencing, aims to characterize the lung microbiome or mycobiome and enables detection of organisms only at the level of genera, thus precise colonization of the specific area with particular microorganisms cannot be determined (Bond et al. 2017, 2020).

Unfortunately, the results of the NGS by Ion Torrent in our study were rather disappointing due to no detection of any fungal DNA. A previous study using this method was successfully performed on BALF samples from cats, which are more concentrated than equine BALF samples, and the samples were frozen only at −20 °C (Načeradská et al. 2021). Therefore, the reason for the failure to detect any fungal DNA in our study could have been inappropriate sampling conditions. The critical point could be the freezing at −80 °C, where highly diluted material is more likely to undergo some degree of degradation. Another problem could be the high dilution of the last portion of BALF retrieved from the lungs, containing too little material which may not be detectable by even so sensitive a diagnostic method. In contrast, the FungiMultiPlex method yielded a positive result in 55% of the cases. Since real-time PCR typically amplifies fragments up to 150 bp (compared with over 400 bp fragments needed for NGS analysis), even low-quality samples can be analysed using this technology.

In several studies, changes in the lung microbiome of asthmatic horses have been proposed to represent a consequence of the disease rather than its cause (Bond et al. 2017; 2020). However, other studies do not exclude the possibility of changes in the microbiome being a contributing factor to exacerbation of clinical signs (Manguin et al. 2020). In human medicine, changes in the composition of the lung microbiome are associated with bronchial hyperresponsiveness and corticosteroid resistance (Huang et al. 2011).

Results of fungal cultivation and FungiMultiPlex were roughly analogous. The association of results of both methods was not confirmed; however, this could be

a consequence of the very low number of individuals on which both tests were performed. For the majority (6/8 horses), the results obtained by both tests were similar. Varying results could have been due to differences in sample storage and shipment. Samples for FungiMultiPlex were stored at −80 °C and shipped with a delay of several days or weeks, depending on the time of sampling. In contrast, samples for fungal cultivation were shipped immediately after sampling and were delivered to the laboratory within 24 h. Both methods concurred on the level of genus with *Aspergillus* spp. being present in all positive samples.

The most commonly detected fungus by both methods was *Aspergillus* spp. We are not aware of any published study which used a similar PCR methodology, so no comparison is possible. *Aspergillus* spp. was isolated in our study in 10/19 (52%) of cases and *Candida* spp. in 1/19 (5%). In a much larger study on a similar population of horses from Western Europe, the most prevalent fungal cultures found were *Penicillium* spp. (53%), followed by *Aspergillus* (34%), *Rhizomucor* (5%), and *Candida* (5%) (Dauvillier et al. 2019). In our study, we did not detect *Penicillium* spp. by any of our methods. Regional differences may play a role, as well as methods (transendoscopic vs. sterile BAL catheter), and also location. Horses in our study were all sampled in the clinic, in the same examination room, whereas horses in the study of Dauvillier et al. (2019) were sampled in the local stables.

Pneumocystis carinii was detected in 3 cases by FungiMultiPlex but not by fungal cultivation because *Pneumocystis* spp. does not grow on standard cultivation media (Schildgen et al. 2014). The number of copies of *Pneumocystis* spp. detected in BALF was low, but given the fact that it is hard to isolate DNA from filamentous fungi, even low quantities of these fungi should be considered relevant.

The limitations of this study were a low number of horses used and a low number of samples where both fungal culture and molecular methods were used, making it impossible to draw any robust statistical conclusion. In addition, no control horses were enrolled. The NGS method is expensive, therefore, it was not possible to try to establish suitable conditions for successful detection of fungal DNA.

Unfortunately, molecular methods are still too expensive to use in clinical routine. On the other hand, knowing the variety of species present in the respiratory fluids from EA horses would allow for the creation of a multiplex panel; the analyses could be less expensive and time consuming, and molecular methods could become a standard procedure in treatment diagnostics.

In conclusion, the results of fungal cultivation and multiplex real-time PCR analysis revealed that fungi were present in approximately half of the BALF samples from asthmatic horses, regardless of the severity of EA. Further studies are needed to elucidate the clinical relevance of the findings and the influence of the environment.

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