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Protein microarray allergen profiling in bronchoalveolar lavage fluid and serum of horses with asthma

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Abstract

Background: The diagnostic value of allergen-specific immunoglobulin E (IgE) in horses with asthma is uncertain. A recently developed protein microarray detected abnormally high latex-specific IgE concentrations in the serum of horses with severe asthma.

Objectives: The main objective was to characterize the IgE profiles of asthmatic horses in Switzerland using a protein microarray platform in serum and bronchoalveolar lavage fluid (BALF). The secondary objective was to determine whether serological and BALF allergen-specific IgE concentrations correlated.

Animals: Forty-four asthmatic and 39 control horses ≥5 years of age.

Methods: This prospective cross-sectional study investigated the sensitization profiles of horses with asthma compared with environmentally matched healthy controls. Both serum and BALF were analyzed using the protein microarray. Partial least square-discriminant analysis (PLS-DA) was used to identify and rank the importance of the allergens for class detection (ie, asthma vs control), with a variable influence on the projection (VIP) >1 considered significant.

Results: The allergens that best discriminated (VIP >1) asthmatic horses from controls were proteins derived from fungi (*Aspergillus fumigatus*), insects (*Culicoides* spp.), and latex (*Hevea brasiliensis*). The serological model predictive ability was markedly inferior (area under the curve [AUC] 0.585, 95% confidence interval [CI]: 0.454-0.747) to that of the BALF (AUC 0.751, 95% CI: 0.582-0.866). The two models shared nine allergens, of which eight showed significant weak to moderate correlations.

Conclusion and Clinical Importance: The concentrations of several allergen-specific IgE were higher in asthmatic horses. The protein microarray performed better on BALF than serum for detection of asthma. Serological IgE concentrations do not closely correlate with BALF concentrations and should be interpreted with caution.

Abbreviations: BALF, bronchoalveolar lavage fluid; CS, clinical score; IgE, immunoglobulin E; PLS-DA, partial least square-discriminant analysis; TW, tracheal wash; VIP, variable influence on the projection.

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KEYWORDS
allergy, fungi, horse, IgE, latex

1 | INTRODUCTION

Asthma is a nonseptic inflammatory disorder of the lower respiratory tract of horses, which encompasses a large spectrum of disease severity. Severe asthma is associated with exercise intolerance, coughing, and increased breathing effort at rest. These clinical signs reflect bronchospasm, increased mucus production, and severe neutrophilic lower airway inflammation that characterize the disease. Manifestations of mild to moderate asthma include decreased performance, cough, and increased tracheal mucus. A relative increase of neutrophil, mastocyte, or eosinophil cell proportions characterizes the BALF cytology of horses affected with mild to moderate asthma, while marked neutrophilic inflammation is a hallmark of severe asthma.[1,2]

Dust exposure reduction is most effective at reducing both severity and frequency of asthma exacerbations. While airborne environmental exposure is known to contribute to the pathogenesis of asthma, the triggering mechanism is not yet elucidated. The lung response to inhaled particulates might be nonspecific (to inhaled irritants such as endotoxins or β-glucans) or directed towards a specific antigen (such as fungi, forage mites, pollen) depending on the proposed allergic (Th-2), non-allergic (non-Th-2), and late-onset endotypes of asthma.[1-4] Research on IgE-mediated reactions in the pathophysiology of asthma has focused on the severe asthma phenotype.[5-11] While these studies did not agree on the role of an allergic component of the disease, there is evidence of higher concentrations of antigen-specific IgE in the serum, BALF, or both of these, of horses with severe asthma.[12-14] Allergens are antigens that elicit immediate-type hypersensitivity reactions. The IgE-binding proteins identified in this study might not all be true allergens. Nonetheless, we use the term "allergen" for all IgE-binding proteins for clarity and consistency with the nomenclature commonly used in this research field. A novel protein microarray using protein extracts as well as purified and recombinant proteins from fungi, pollen, bacteria, arthropods, and other potential allergen sources in the horse environment allows large-scale profiling of allergen-specific IgEs (n = 384) in the serum and BALF from horses affected of severe asthma compared with controls.[13] The sensitization profile from severely asthmatic horses comprised mainly allergens issued from latex, fungi, mite, and pollen.[13] The detection of high latex-specific IgE concentrations in the serum of severely asthmatic horses[12,13] demonstrates the potential of this expanded protein microarray to interrogate the immunological mechanisms of asthma in horses. In contrast, a commercially available screening test with a limited allergen panel (Allercept, Heska Allergy Products) revealed no or limited association with the asthma status of the horses and allergen-specific serum IgE concentrations.[11,14]

The main aim of this study was to characterize the allergen-specific IgE profile of asthmatic horses in Switzerland using the recently developed protein microarray platform in both BALF and serum. Horses without clinical signs living in the same environment served as controls. The second aim was to determine whether systemic allergen-specific IgE concentrations reflected lung local production by investigating the agreement between BALF and serum concentrations. We hypothesized that microarray profiling would allow for discrimination between asthmatic and control horses in a field situation based on their allergen-specific IgE profiles. We anticipated that serological and BALF allergen-specific IgE concentrations would be significantly correlated.

2 | MATERIAL AND METHODS

2.1 | Study design, recruitment of horses, and owner questionnaire

The study was designed as an analytical, observational, prospective case-control study. All procedures were performed in a veterinary medical teaching hospital (ISME equine clinic, University of Bern). The experimental design and methods were approved by the Animal Experimentation Committee of the Canton of Bern (BE4/20+). Informed client consent was obtained for all horses. The study was advertised on social media. Horses were also recruited through referring veterinarians. Suitable candidates were identified using the validated horse-owner assessed respiratory signs index (HOARSI).[15] Briefly, horses were graded on a scale from 1 to 4 based on the owner-reported signs of respiratory disease (coughing, nasal discharge, breathing pattern and performance). These grades represent healthy (grade 1) to severely affected (Grade 4) individuals. Horses with a HOARSI score ≥2 were included as cases. One horse without clinical signs (HOARSI = 1) served as an environmentally-matched control for 1 or several asthmatic horse(s) of the same stable. Inclusion and exclusion criteria are listed in Table S1.

2.2 | Clinical examination

Two veterinarians (MW, SS) performed all examinations and procedures on the horses. Horses were weighted and brought to a dust-free stall. They underwent complete physical examination after they had settled. Specific evaluation of the respiratory system was performed using a validated clinical scoring system.[16,17] Abdominal lift (normal, mild, pronounced), nasal flare (none, present), cough (none, inducible by massage, intermittent, paroxysmal), tracheal sounds (normal, increased, mucus movement [rattles]), bronchial tones (normal, audible ventral and dorsal sounds), crackles (none, present), and wheezes (none, present) were assessed when the horse was spontaneously breathing. A rebreathing examination was then performed.

2.3 | Blood collection, tracheal mucus scoring, tracheal wash, and BALF collection

Venous blood (serum Monovette tube, Sarstedt, Nümbrecht, Germany) was collected via direct aseptic venipuncture. Horses were sedated with detomidine 0.01 mg/kg IV (Equisedan, Graeub, Bern, Switzerland) and butorphanol 0.01 to 0.02 mg/kg IV (Morphasol-10, Graeub, Bern, Switzerland). An endoscope (VET-OR1200HD, Medical Solution GMBH, Wil, Switzerland) was passed into the trachea down to the carina. The amount of tracheal mucus was assessed using a validated scoring system.[18] Ten milliliter of sterile 0.9% NaCl were instilled through a Teflon-coated PVC catheter and immediately reaspirated. A sterile BALF tube (either 240 or 300 cm long depending on horse size; 10 mm outer diameter; Bivona Medical Technologies, Gary, IN) was inserted through the nose and into the trachea. The volumes of lidocaine (Lidocain 2% Streuli, Streuli Pharma AG, Uznach, Switzerland) and 0.9% NaCl used for the BALF were determined based on allometric scaling (Table S2). The predetermined volume of lidocaine was instilled and the tube was advanced until wedged. The cuff was inflated with 5 mL of air. The predefined volume of warm sterile 0.9% NaCl was instilled and reaspirated using 60-mL syringes. The cuff was deflated before tube removal. The BALF was pooled in an iced silicone-coated glass bottle (Fisher Scientific, Leicestershire, UK). The BALF yield was defined as the ratio of volume recovered to volume instilled. If the BALF yield was ≤20%, or if the BALF quality was poor (no foam), the procedure was repeated a second time.

2.4 | Sample processing

The BALF was filtered through a 40 μm cell strainer (Falcon, Fisher Scientific, Leicestershire, UK). Direct smears of the tracheal wash (TW) were prepared within 15 minutes of collection and quickly dried with a cold hair dryer. Cytospins were performed within 2 hours of collection by centrifugation during 5 minutes at 800 rpm (Hettich Rotofix 32, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Direct smears and cytospin slides were stained with a modified Wright-Giemsa. A single investigator (SS) performed the BALF differential counts (macrophages, lymphocytes, neutrophils, eosinophils, and mast cells) using a minimum of 400 cells and four different microscopic fields at ×1000 magnification with oil immersion. A semi-quantitative score for the amount of neutrophils was attributed to each TW slide (0: none, 1: mild, 2: moderate, 3: marked). The two 9 mL serum tubes were gently inverted 10 times and stored in a vertical position at room temperature for ~30 minutes. The tubes were then centrifuged at 2000*g* for 10 minutes (Eickemeyer PLC-02, Eickemeyer - Medizintechnik für Tierärzte KG, Tuttlingen, Germany). The serum obtained was transferred into four 1.5 mL cryotubes (Eppendorf AG, Hamburg, Germany) and stored at −80°C.

The BALF was centrifuged at 1500 rpm for a total of 5 minutes at +4°C. A Pierce Protease Inhibitor Mini tablet (product # 13437766) was added to 10 mL of the supernatant. The sample was gently agitated until complete dissolution of the tablet and 2.5 mL glycerol was added. The samples were then stored at −80°C until processing in an Amicon Ultra-15 centrifugal filter (product # UFC905024) to a 40-fold concentration, as described by White et al.[13]

Both serum and BALF samples were shipped on dry ice to Nottingham Trent University, UK, for microarray profiling.

2.5 | Protein microarray

A comprehensive complex microarray comprised of protein extracts (n = 153) and pure proteins (n = 231) from a wide range of fungi, bacteria, pollen, arthropods, and others found in the typical horse environment was used, as described in White et al.[12,13] In these two studies, sample preparation optimization, validation with ELISA and mathematical cross-validation have been explained in detail. Briefly, normalized proteins were printed onto ONCYTE NOVA Nitrocellulose Film Slides (Grace Bio-Labs, OR) using an Ultra Marathon II by Arrayjet (Roslin, Scotland). Slides were blocked in 3% BSA, fitted with Proplate slide modules and hybridized with serum and BALF samples from the horses tested diluted 1:2 overnight at +4°C. Slides were subsequently incubated with a monoclonal mouse anti-horse IgE (BioRad, #MCA5982GA) and DyLight 649 conjugated anti mouse IgG (Rockland, Product #610-443-040) before washing and drying via centrifugation.

Slides were then scanned in a GenePix 4000B (Molecular Devices, USA) and processed using GenePix Pro software v6.0.1.27 (Axon Instruments). Digital fluorescence units (DFUs) were calculated for each spot by subtracting local background from the median fluorescence value of the spot. One pad on each microarray was used as a control, containing reagents and no sample, the results of which were subtracted from all other pads to account for any auto-fluorescence or nonspecific binding.

2.6 | Statistical analyses

All analyses were performed using R Studio 1.4.1717. Normality of the descriptive data (study sample characteristics) was assessed using the Kolmogorov-Smirnov test. The differences between asthmatic and control groups of horses were analyzed using the Wilcoxon signed-rank test. A partial least square-discriminant analysis (PLS-DA) was performed to separate predefined classes (ie, asthmatic/control) to identify and rank the importance of different variables (allergens) in class prediction. PLS-DA is a supervised classification method used to establish separation between different groups of samples, which is completed by linking two data matrices *X* (ie, the protein DFUs) and *Y* (asthmatic/control groups). A variable influence on the projection (VIP) score of each variable was calculated as a weighted sum of the squared correlations between the original variable and the PLS-DA components. The VIP reflects the contribution of a specific variable and is therefore a measure of a variable's importance within the model. The significance threshold was set to VIP >1, and all proteins exceeding this value are referred to as allergens in the text. Receiver operating characteristic (ROC) curve of each PLS-DA model was used to predict class outcome. Confidence intervals were calculated by Monte Carlo Cross Validation (MCCV)

using balanced subsampling with multiple repeats. MCCV creates multiple random splits of the dataset into training and validation data. For each split, the model is fitted to the training data, and predictive accuracy is assessed using the validation data. The results are then averaged over the splits. All proteins selected for the second round of modeling were included. In each MCCV, two thirds (2/3) of the samples are used to evaluate the feature importance. The top important features are then used to build the classification model which is validated on the 1/3 of the samples that were left out. The procedure was repeated multiple times to calculate the performance and confidence interval of each model.

Spearman's Rank Correlation Coefficients of specific IgE fluorescence values for all proteins (n = 384), as well as common VIPs between BALF vs serum PLS-DA models, were used to assess the relationship between BALF and serum.

3 | RESULTS

3.1 | Horses

Eighty-six horses were examined for the study, three of which were excluded. One horse had numerous hemosiderophages in the BALF, suggesting previous pulmonary hemorrhage. This horse was excluded because asthma was most likely not the primary cause for its respiratory signs. Its environmentally-matched control was also removed from the study. Another asthmatic horse was excluded because of an insufficient BALF yield despite repeating the procedure. Its matched control remained in the study, as it served as a control for two other asthmatic horses from the same stable. A total of 83 horses (controls n = 39, asthmatic horses n = 44) were included in the final analysis. In accordance with the inclusion criteria, all control horses had a HOARSI of 1, and all cases had a HOARSI ≥2 (20 with HOARSI = 2, 7 with HOARSI = 3, and 17 with HOARSI = 4). These horses came from 39 different stables: 35 stables with a case/control pair, 3 stables with 1 control for 2 cases, and 1 stable with 1 control for 3 cases. Represented breeds in the study sample were Warmblood (n = 36), American Quarter Horse (n = 9), Freiberger (n = 7), Welsh Cob Pony (n = 6), Islandic Pony (n = 6), Arabian (n = 4), Shetland Pony (n = 4), Haflinger (n = 3), Purebred Spanish Horse (n = 3), Lusitano (n = 2), and Standardbred (n = 2).

3.2 | Clinical examination

The clinical score (CS), the tracheal mucus score, the tracheal neutrophil score and the relative distribution of neutrophils in the BALF differed significantly between groups (Table 1).

3.3 | BALF samples

The initial BALF calibration of the PLS-DA classification method was performed by incorporating all proteins included in the microarray.[13] In an effort to reduce the background noise and to improve the robustness of the mathematical model, a second round of modeling was conducted using the main VIPs (n = 65) identified in the calibration step (Figure 1A), as described in White et al.[13] This improved the overall accuracy of the mathematical model (Figure 1B).

Among the 18 allergens retained in the final model, 17 (94%) showed increased specific IgE concentrations in asthmatic compared with control horses. Based on the area under the curve (AUC) value of the PLS-DA calibration model (AUC 0.751, 95% confidence interval [CI]: 0.582-0.866), asthmatic horses could be discriminated from controls using the microarray platform on BALF.

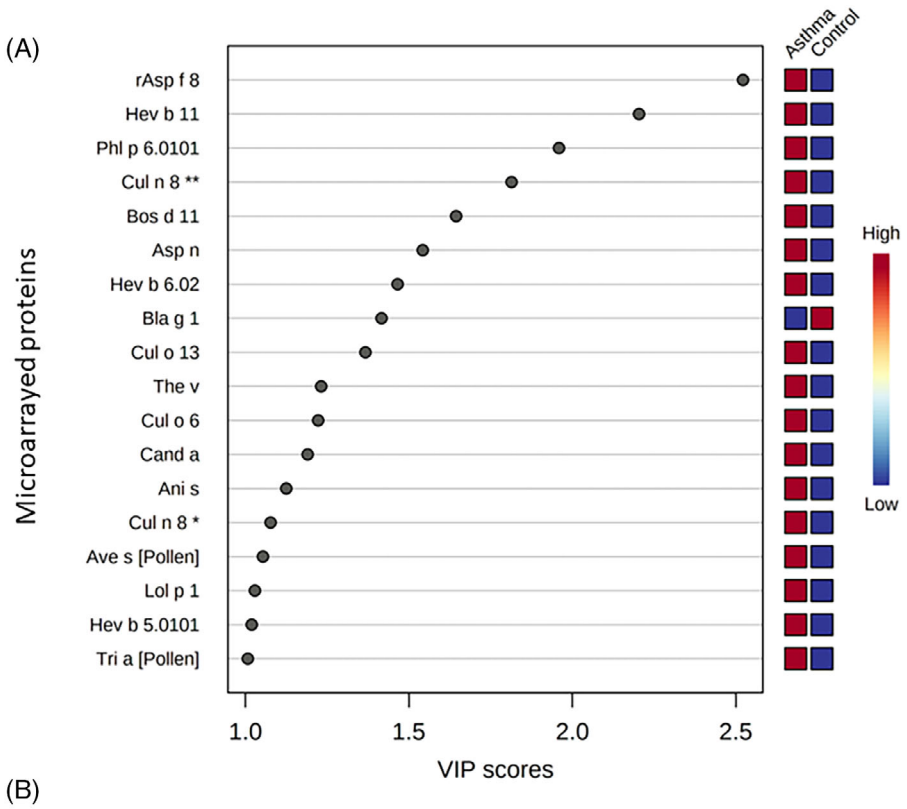
3.4 | Serum samples

A total of 23 allergen-specific IgEs were retained in the final model. The predictive ability of the serological model was mostly

TABLE 1 Characteristics of the study sample of horses

	Control			Asthmatic			
	Median	Range	IQR	Median	Range	IQR	*P* value
Age (years)	12.12	23.55	8.79	14.33	19.97	9.38	.51
Body weight (kg)	542.00	618.00	108.00	517.00	467.00	161.50	.05
CS (1-23)	1.00	5.00	2.00	5.00	14.00	5.00	<.0001
BALF yield (%)	52.00	61.33	16.00	50.00	47.00	20.00	.54
TM score (1–5)	1.00	3.00	2.00	3.00	5.00	2.00	<.0001
TW neu (%)	.00	3.00	1.00	1.00	3.00	2.00	<.0001
BAL mac (%)	39.75	50.25	17.50	34.88	62.75	23.75	.26
BAL lym (%)	50.00	47.25	13.00	50.00	73.25	20.25	.20
BAL neu (%)	7.00	25.50	6.50	13.38	73.50	17.75	.02
BAL mast (%)	1.50	4.75	1.75	1.75	7.25	2.38	.76
BAL eos (%)	.00	11.25	.50	.00	22.75	.25	.99

Abbreviations: BAL eos, BAL eosinophils (BAL eos); BAL lym, BAL lymphocytes; BAL mac, BAL macrophages; BAL mast, BAL mastocytes; BAL neu, BAL neutrophils; CS, 23-point-weighted-clinical score; TM score, tracheal mucous score; TW neu, tracheal wash neutrophils.



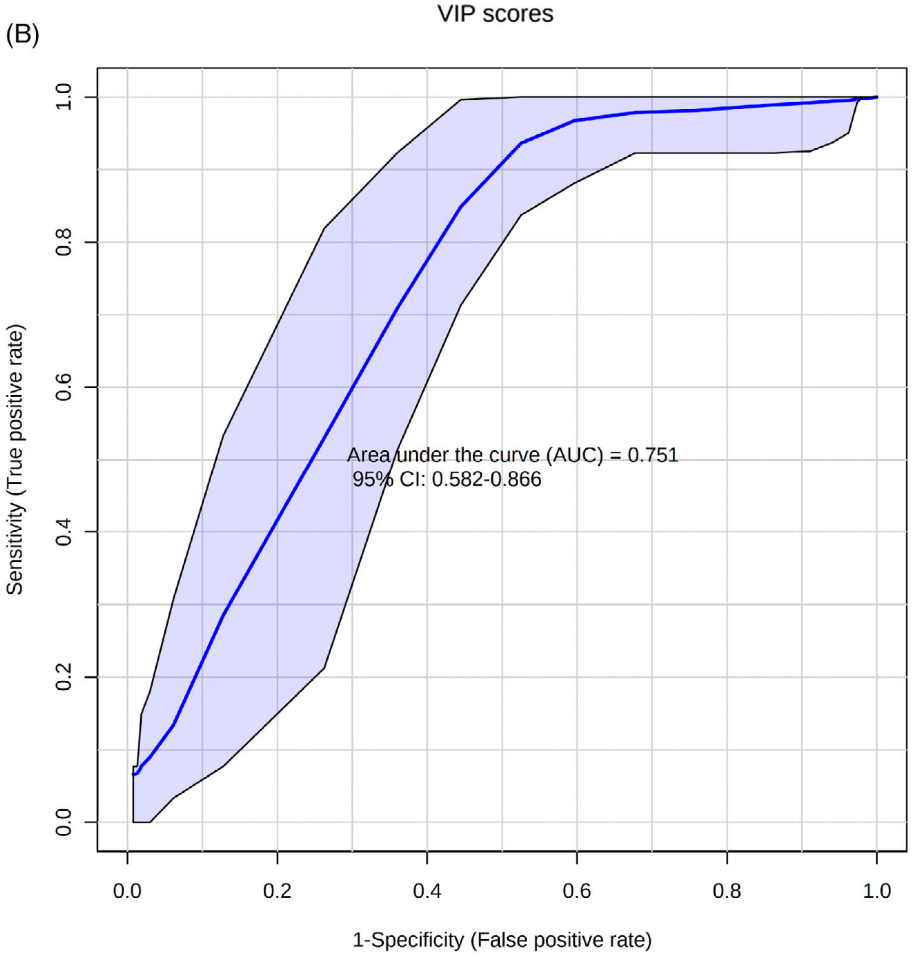


FIGURE 1 Results of statistical analyses for the bronchoalveolar lavage samples. (A) Variable influence on the projection (VIP) of the proteins included in the microarray calculated by partial least square-discriminant analysis (PLS-DA) after VIP selection. The significance threshold was set to VIP >1. High-low scale refers to IgE quantity based on fluorescent units. * expressed in yeast; ** expressed in *E. coli*. (B) Receiver operating characteristics (ROC) curve based on the cross-validation performance with a 95% confidence interval computed for the PLS-DA model

based on 14 allergens with higher concentrations in controls compared with cases. Only 9 out of 23 allergen-specific IgEs (39%) were increased in asthmatic horses compared with controls (Figure 2A).

The predictive accuracy of the PLS-DA calibration modeling for discrimination of cases from controls was low (AUC 0.585, 95% CI: 0.454-0.747), even after a second round of modeling using VIP selection (n = 76) (Figure 2A,B).

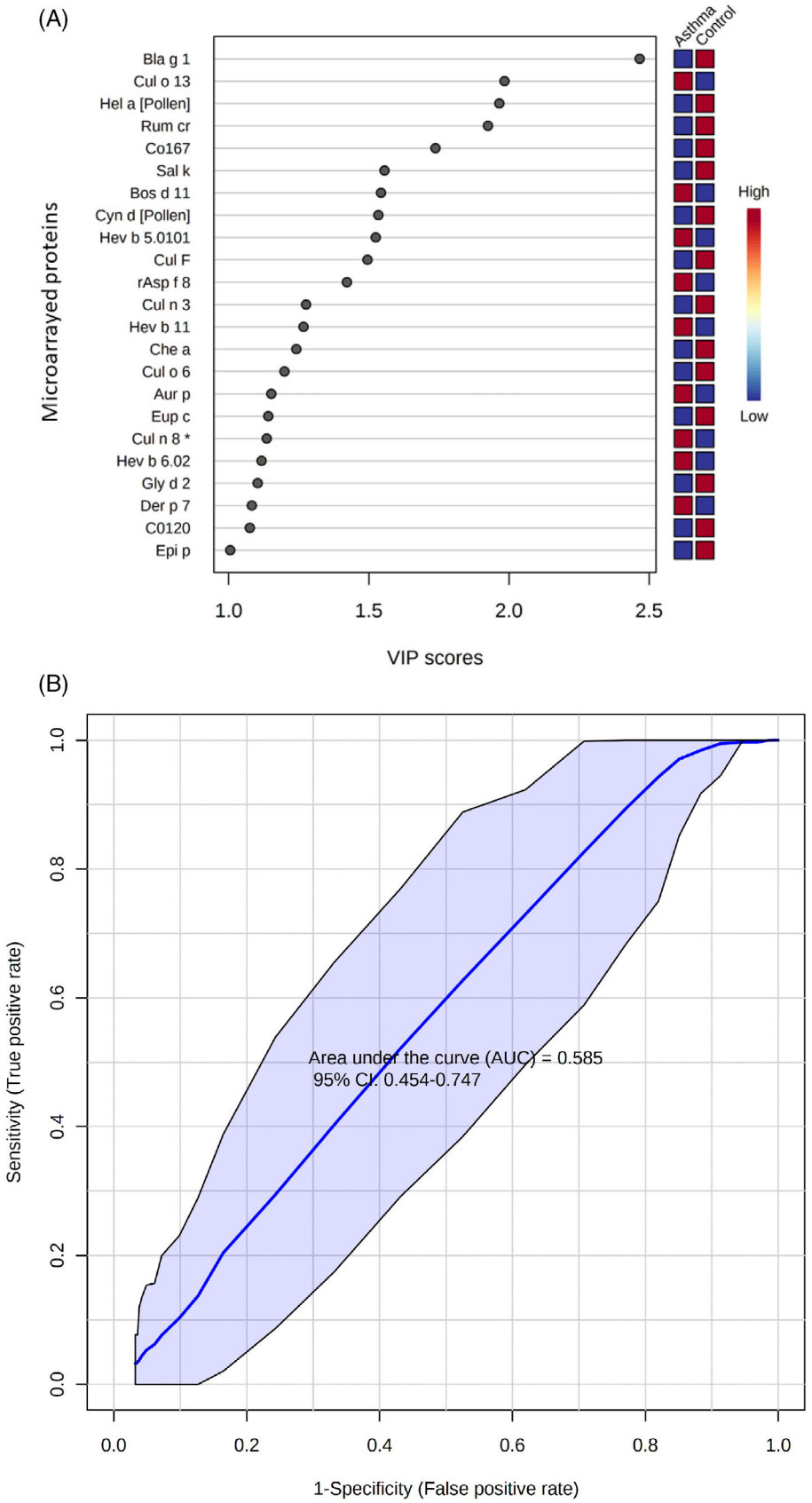


FIGURE 2 Results of statistical analyses for the serum samples. (A) Variable influence on the projection (VIP) of the proteins included in the microarray calculated by partial least square-discriminant analysis (PLS-DA) after VIP selection. A threshold of $\alpha > 1$ was used to identify the VIPs significant in class detection. High-low scale refers to IgE quantity based on fluorescent units. * expressed in yeast; ** expressed in *E. coli*. (B) Receiver operating characteristics (ROC) curve based on the cross-validation performance with a 95% confidence interval computed for the PLS-DA model

A wide range of allergen-specific IgEs were identified as significant for class prediction (asthmatic vs control) in both the BALF and serological models (n = 18 and n = 23, respectively). Allergens common to both models included rAsp f 8, Hev b 11, Hev b 5.0101, Hev b 6.02, Bla g 1, Bos d 11, Cul o 13, Cul n 8, and Cul o 6. Of note, Bla g 1 was significant for class prediction in both models because of higher

TABLE 2 Correlation (Spearman's rank test) between specific allergen IgE levels in BALF and serum for the allergens retained in both BALF and serum final PLS-DA models

Allergen	Expression system	r_s value	*P* value
Bla g 1	*E. coli*	.78	<.0001
Bos d 11	*E. coli*	.44	<.0001
Cul o 13	*E. coli*	.47	<.0001
Cul n 8	*Pichia pastoris* (yeast)	.47	<.0001
Cul o 6	*Pichia pastoris* (yeast)	.05	.66
Hev b 11	*E. coli*	.32	<.01
Hev b 5.0101	*E. coli*	.32	<.01
Hev b 6.02	*E. coli*	.41	<.001
rAsp f 8	*E. coli*	.47	<.0001

concentrations in controls compared with asthmatic horses. The predictive ability of the PLS-DA model was markedly better when using BALF compared with serum, based on a higher AUC (see Figures 1B and 2B).

3.5 | Comparison of allergen-specific IgE concentrations in BALF and serum

Spearman's rank correlation coefficients demonstrated a weak correlation ($r_s = .39$) between BALF and serum allergen-specific IgE fluorescence values when all proteins were combined (n = 384). Individual correlations were then calculated for the nine allergens retained in both BALF and serum PLS-DA models. The serum and BALF concentrations were found to be significantly correlated for eight allergens (Table 2). Correlation was strong ($r_s = .6$-$.79$) or moderate ($r_s = .4$-$.59$) for the majority of these allergens, but only weak ($r_s < .4$) for Hev b 11 and Hev b 5.0101 (Table 2).

4 | DISCUSSION

In this study, we characterized the allergen-specific sensitization profiles of a field sample of asthmatic horses in Switzerland, using a recently developed protein microarray on BALF and serum. The asthmatic status of a horse could be detected using the microarray on BALF. As expected, most of the significant allergen-specific IgEs had higher concentrations in asthmatic horses. In contrast, serological testing did not discriminate between asthmatic and control horses with an acceptable level of certainty. This was attributed to the fact that most of the significant allergen-specific IgEs were higher in controls, and not in asthmatic horses as expected. When investigating asthma in horses, serological IgE concentrations are probably less informative than BALF's, which reflect the lung local allergic response. Serological IgE concentrations likely indicate frequent environmental exposure rather than true sensitization, and thus might not be suitable for disease prediction. However, the results of BALF microarray cannot be interpreted alone, because of the potential bias introduced by uncontrolled BALF dilution. Indeed, IgE concentrations are affected by the degree of BALF dilution, which varies with BALF return. In the absence of an internal standard (similar to creatinine for urine analyses), IgE concentrations cannot be calibrated. Furthermore, filtering of BALF could result in loss of specific IgE trapped in mucus, but is necessary for sample processing.[13] Consequently, we focused on the nine allergens that were significant in both the serological and BALF models.

The common IgE profile obtained with the serological and BALF models revealed sensitization to allergens derived from fungi, insects, and latex. Similar sensitization profiles are found in both BALF and serum of severely asthmatic horses, using the same protein microarray.[13] A study performed with a commercial ELISA screening test identified increased allergen-specific BALF IgE to insects, mites, and pollen in horses affected with mastocytic or mixed mild to moderate asthma, but not with other subtypes of asthma in horses.[14] Among the nine allergens retained in both the BALF and serum models was *Aspergillus fumigatus* (rAsp f 8). Sensitization to this recombinant mold allergen appears to be a consistent feature of asthma, with increased IgE concentrations previously detected using the same microarray[12,13] or other techniques.[9,11,19] There are significantly higher positive intradermal reactions to rAsp f 8 in severely asthmatic horses.[8]

Besides *Aspergillus fumigatus*, three of the most influential allergens for disease detection in our study were those derived from latex (*Hevea brasiliensis*, Hev b). Elevation of Hev b 11 and Hev b 6.02-specific IgE are described in severely asthmatic horses.[12] We confirmed these results in a field sample of horses affected with predominantly neutrophilic mild to moderate asthma (based on low clinical scores). Latex is an allergic trigger in people, in particular among healthcare workers. Urticaria, as well as systemic anaphylactic and asthmatic reactions, correlate with higher latex-specific serum IgE concentrations.[20] The role of latex has been questioned, because of potential cross-reactivity with fruit or grass pollen (*Phleum pratense*, Phl p 12) antigens.[21,22] Therefore, patients with high latex-specific serum IgE concentrations should undergo additional diagnostic testing such as a skin prick test to confirm a type I hypersensitivity reaction to latex allergens.[21] So-called cross-reactive carbohydrate determinants, which occur mainly in allergens produced in yeast, increase the likelihood of cross-reactivity.[20] Since the latex, *Aspergillus fumigatus* and most of the *Culicoides* spp. proteins used in the protein microarray were expressed in *Escherichia coli*, cross-reactions against carbohydrate determinants should not be of concern in the present study. Future investigations should determine whether the observed latex sensitization in horses with asthma is clinically relevant, for example, with functional assays such as cellular allergen stimulation test or intrabronchial challenge, and whether cross-reactivity occurs. If cross-reactivity can be dismissed, the source of latex exposure should be further investigated. For healthcare workers, latex gloves are thought to be the main source of exposure.[23] *Hevea brasiliensis*, the latex tree, does not grow naturally, nor is it cultivated, in our geographical location. Possible culprits in the horse environment entail footing products in arenas and paddocks, rubber mats used in stables, or suspended particles originating from tire abrasion nearby high-traffic roads.

Out of the nine discriminant allergens in BALF and serum, three were from *Culicoides*-derived allergens (*Culicoides obsoletus* Cul o 13, Cul o 6 and *Culicoides nubeculosus* Cul n 8). Of note, none of the asthmatic horses in the present study had a history of insect bite hypersensitivity (IBH). Interestingly, it was previously shown that horses suffering from asthma were at higher risk of developing IBH.[24] On the other hand, IBH-affected horses without clinical signs of asthma showed greater airway hyperreactivity.[25] The observed sensitization to some *Culicoides* r-allergens might thus reflect a predisposition for IBH in horses with asthma. A recent study, however, did not find increased *Culicoides*-specific serum IgE concentrations in horses with asthma.[26] The *Culicoides* r-allergens Cul o 13, Cul o 6, and Cul n 8 were, however, not included in the latter study, which could explain this discrepancy. More experiments are needed to determine whether the observed sensitization is primarily directed towards allergens from *Culicoides* or results from cross-reactivity with yet unidentified allergens. Cul o 6 and Cul o 13 are both D7/odorant binding proteins,[27] while Cul n 8 is a maltase.[28] More than 25% of the proteins printed on the protein microarray consisted of *Culicoides* proteins. This overrepresentation could explain in part the high proportion of *Culicoides* proteins retained in the final model.

Among the nine allergens retained in both the BALF and serum models only German cockroach *Blatella germanica* (Bla g 1) had higher IgE concentrations in control horses. High Blatella-specific serum IgE concentrations are reported in healthy and asthmatic horses.[12,13] A likely explanation is the common and frequent exposure to cockroaches in barns and feedstuffs.

While the BALF and serum IgE concentrations were significantly correlated for eight of the nine discriminating allergens retained in the final model, only 1 (Bla g 1) showed a strong linear relationship. The concentrations of the seven other allergens were only moderately or weakly correlated despite statistical significance. So far, only two studies have investigated allergen-specific IgE concentrations in the BALF of asthmatic horses. Using the same protein microarray, there is good agreement between BALF and serum in severely asthmatic horses.[13] This might be explained by the differences in the samples studied. The horses included in the present study had relatively low clinical scores, suggesting they either were affected by a mild form of asthma or were in partial clinical remission from severe asthma. One may postulate that in severely asthmatic horses, the strong sensitization at the lung level is better reflected systemically, compared with horses with milder forms of the disease. There are no significant correlations between serum and BALF for any of the allergens screened in horses with various subtypes of mild to severe asthma.[14] However, the commercial ELISA test they used comprised a more limited screening panel with proteins of various types and sources (eg, recombinant proteins vs extracts), preventing direct comparison with the results of our microarray study. To summarize, we found that BALF IgE concentrations did not correlate strongly with serum IgE concentrations, suggesting that serological screening test in horses with asthma should be interpreted with caution.

This study has several limitations. Horses were defined as control or case based on their HOARSI. Lung function testing was not available for asthma status confirmation. Horses with a HOARSI of 3 or 4 were expected to show a CS >14.[16] Yet, asthmatic horses within our study sample had CS ranging from 1 to 14, indicating mild to moderate disease severity.[16] The 23-point CS discriminates well between mild or subclinical cases and severe cases. It is, however, not sensitive enough to differentiate mildly to moderately asthmatic horses from clinical remission of severe asthma.[4,16] Moreover, the HOARSI is based on the horse's respiratory signs at its worst, but does not necessarily reflect disease severity at the time of inclusion. Owners of the asthmatic cases were aware of their condition and had improved the management accordingly, which likely explains their mild to moderate clinical signs. Overall, our case sample was composed of horses affected with asthma of varying severity, with predominantly mild to moderate neutrophilic asthma. Besides the CS, all horses underwent bronchoscopy, TW and BAL to assess their lower airway inflammation status. Clinical score, tracheal mucus score, tracheal neutrophil score and BALF neutrophil percentage were significantly different between groups, suggesting horses were appropriately assigned to the asthmatic and control groups. In the absence of a more sensitive diagnostic tool such as lung function testing, we cannot exclude that some horses were misclassified. Indeed, some of the control horses showed cytological findings compatible with asthma (eg, increased BALF neutrophil percentages) based on the ACVIM consensus statement.[2] Nonetheless, horses included in this study are representative of the Swiss horse population, which should facilitate the translation of the results to field practice.

Environmental matching was imperfect. Since environmental exposures determine if and how robustly horses may produce allergen-specific IgE concentrations,[19] we decided to match each asthmatic horse with a control horse from the same stable. Each control was matched with a maximum of three cases to prevent a stable bias. Even though matched horses were housed in the same stable, other management factors differed (eg, feed type or bedding). The variable environmental exposures may thus have influenced the IgE concentrations.

The cross-sectional design of the present study has inherent predictive limitations, since a temporal and potentially causal link between increased allergen-specific IgE concentrations and manifestation of asthma on horses cannot be established. A recent intradermal testing study advocated a predominant involvement of type I hypersensitivity in severe asthma (based on immediate reactions), while type IV hypersensitivity seemed to play a more important role in mild to moderate asthma (based on delayed reactions).[29] Studies specifically targeting cases belonging to the mastocytic type of mild to moderate asthma[14] or the suspected allergic endotype of severe asthma[3] might provide further insight into the role of IgE in asthma and the clinical usefulness of allergen screening. Longitudinal studies might also provide crucial information on the possible seasonality of asthma and its associated sensitization profiles.

This prospective cross-sectional study investigated the sensitization profiles of a field sample of horses predominantly affected with mild to moderate neutrophilic asthma and their

environmentally-matched controls using an extensive protein microarray. Among the allergens relevant for class detection, nine were significant in both BALF and serological IgE profiles. These included allergens derived from fungi, *Culicoides*, and latex. Our findings support previous observations that BALF IgE concentrations might be more informative than serum IgE concentrations in horses with asthma. The value of BALF allergen-specific IgE concentrations as a diagnostic tool deserves further investigation. The identification of potential allergenic triggers for asthma in horses could guide future therapies (eg, desensitization) and preventative measures (eg, avoidance of a specific allergen exposure).

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Animal Experimentation Committee of the Canton of Bern, Switzerland (BE4/20+).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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REFERENCES

1. Leclere M, Lavoie-Lamoureux A, Lavoie J-P. Heaves, an asthma-like disease of horses. *Respirology*. 2011;16:1027-1046.
2. Couëtil LL, Cardwell JM, Gerber V, Lavoie JP, Léguillette R, Richard EA. Inflammatory airway disease of horses—revised consensus statement. *J Vet Intern Med*. 2016;30:503-515.
3. Bond S, Léguillette R, Richard EA, et al. Equine asthma: integrative biologic relevance of a recently proposed nomenclature. *J Vet Intern Med*. 2018;32:2088-2098.
4. Couëtil L, Cardwell JM, Leguillette R, et al. Equine asthma: current understanding and future directions. *Front Vet Sci*. 2020;7:450.
5. Halliwell RE, McGorum BC, Irving P, Dixon PM. Local and systemic antibody production in horses affected with chronic obstructive pulmonary disease. *Vet Immunol Immunopathol*. 1993;38:201-215.
6. Schmallenbach KH, Rahman I, Sasse HH, et al. Studies on pulmonary and systemic aspergillus fumigatus-specific IgE and IgG antibodies in horses affected with chronic obstructive pulmonary disease (COPD). *Vet Immunol Immunopathol*. 1998;66:245-256.
7. van der Haegen A, Griot-Wenk M, Welle M, et al. Immunoglobulin-E-bearing cells in skin biopsies of horses with insect bite hypersensitivity. *Equine Vet J*. 2001;33:699-706.
8. Tahon L, Baselgia S, Gerber V, et al. In vitro allergy tests compared to intradermal testing in horses with recurrent airway obstruction. *Vet Immunol Immunopathol*. 2009;127:85-93.
9. Künzle F, Gerber V, van der Haegen A, Wampfler B, Straub R, Marti E. IgE-bearing cells in bronchoalveolar lavage fluid and allergen-specific IgE levels in sera from RAO-affected horses. *J Vet Med A*. 2007;54:40-47.
10. Scharrenberg A, Gerber V, Swinburne JE, et al. IgE, IgGa, IgGb and IgG (T) serum antibody levels in offspring of two sires affected with equine recurrent airway obstruction. *Anim Genet*. 2010;41:131-137.
11. Klier J, Lindner D, Reese S, Mueller RS, Gehlen H. Comparison of four different allergy tests in equine asthma affected horses and allergen inhalation provocation test. *J Equine Vet*. 2021;102:103433.
12. White SJ, Moore-Colyer M, Marti E, et al. Antigen array for serological diagnosis and novel allergen identification in severe equine asthma. *Sci Rep*. 2019;9:1-8.
13. White S, Moore-Colyer M, Marti E, et al. Development of a comprehensive protein microarray for immunoglobulin E profiling in horses with severe asthma. *J Vet Intern Med*. 2019;33:2327-2335.
14. Hansen S, Otten ND, Birch K, Skovgaard K, Hopster-Iversen C, Fjeldborg J. Bronchoalveolar lavage fluid cytokine, cytology and IgE allergen in horses with equine asthma. *Vet Immunol Immunopathol*. 2020;220:109976.
15. Ramseyer A, Gaillard C, Burger D, et al. Effects of genetic and environmental factors on chronic lower airway disease in horses. *J Vet Intern Med*. 2007;21:149-156.
16. Lavoie J-P, Bullone M, Rodrigues N, Germim P, Albrecht B, Von Salis-Soglio M. Effect of different doses of inhaled ciclesonide on lung function, clinical signs related to airflow limitation and serum cortisol levels in horses with experimentally induced mild to severe airway obstruction. *Equine Vet J*. 2019;51:779-786.
17. Pirie RS, Mueller H-W, Engel O, Albrecht B, von Salis-Soglio M. Inhaled ciclesonide is efficacious and well tolerated in the treatment of severe equine asthma in a large prospective European clinical trial. *Equine Vet J*. 2021;53:1094-1104.
18. Gerber V, Straub R, Marti E, et al. Endoscopic scoring of mucus quantity and quality: observer and horse variance and relationship to inflammation, mucus viscoelasticity and volume. *Equine Vet J*. 2004;36:576-582.
19. Eder C, Crameri R, Mayer C, et al. Allergen-specific IgE levels against crude mould and storage mite extracts and recombinant mould allergens in sera from horses affected with chronic bronchitis. *Vet Immunol Immunopathol*. 2000;73:241-253.
20. Nowakowska-Świrta E, Wiszniewska M, Walusiak-Skorupa J. Allergen-specific IgE to recombinant latex allergens in occupational allergy diagnostics. *J Occup Health*. 2019;61:378-386.
21. Brandi SL, Poulsen LK, Garvey LH. The clinical relevance of natural rubber latex-specific IgE in patients sensitized to timothy grass pollen. *Int Arch Allergy Immunol*. 2019;178:345-354.
22. Giangrieco I, Ricciardi T, Alessandri C, et al. ENEA, a peach and apricot IgE-binding protein cross-reacting with the latex major allergen Hev b 5. *Mol Immunol*. 2019;112:347-357.
23. Filon FL, Radman G. Latex allergy: a follow up study of 1040 healthcare workers. *Occup Environ Med*. 2006;63:121-125.
24. Kehrli D, Jandova V, Fey K, Jahn P, Gerber V. Multiple hypersensitivities including recurrent airway obstruction, insect bite hypersensitivity, and urticaria in 2 warmblood horse populations. *J Vet Intern Med*. 2015;29:320-326.

25. Lanz S, Brunner A, Graubner C, Marti E, Gerber V. Insect bite hypersensitivity in horses is associated with airway hyperreactivity. *J Vet Intern Med*. 2017;31:1877-1883.
26. Verdon M, Lanz S, Rhyner C, Gerber V, Marti E. Allergen-specific immunoglobulin E in sera of horses affected with insect bite hypersensitivity, severe equine asthma or both conditions. *J Vet Intern Med*. 2019;33:266-274.
27. Novotny EN, White SJ, Wilson AD, et al. Component-resolved microarray analysis of IgE sensitization profiles to Culicoides recombinant allergens in horses with insect bite hypersensitivity. *Allergy*. 2021;76:1147-1157.
28. Schaffartzik A, Marti E, Torsteinsdottir S, Mellor PS, Crameri R, Rhyner C. Selective cloning, characterization, and production of the Culicoides nubeculosus salivary gland allergen repertoire associated with equine insect bite hypersensitivity. *Vet Immunol Immunopathol*. 2011;139:200-209.
29. Lo Feudo CM, Stucchi L, Alberti E, Conturba B, Zucca E, Ferrucci F. Intradermal testing results in horses affected by mild-moderate and severe equine asthma. *Animals*. 2021;11:2086.

SUPPORTING INFORMATION

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

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