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Microarray molecular mapping of horses with severe asthma

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Abstract

Background: Severe asthma (SA) in horses, resembling human asthma, is a prevalent, debilitating allergic respiratory condition marked by elevated allergen-specific immunoglobulin E (IgE) against environmental proteins; however, research exploring the exposome's influence on IgE profiles is currently limited but holds paramount significance for diagnostic and therapeutic developments.

Animals: Thirty-five sports horses were analyzed, consisting of environmentally matched samples from France (5 SA; 6 control), the United States (6 SA; 6 control), and Canada (6 SEA; 6 control).

Methods: This intentional cross-sectional study investigated the sensitization profiles of SA-affected and healthy horses via serological antigen microarray profiling. Partial least square-discriminant analysis (PLS-DA) was used to identify and rank the importance of allergens for class separation (ie, affected/non-affected) as variable influence of projection (VIP), and allergen with commonality internationally established via frequency analysis.

Results: PLS-DA models showed high discriminatory power in predicting SA in horses from Canada (area under the curve [AUC] 0.995) and France (AUC 0.867) but poor discriminatory power in horses from the United States (AUC 0.38). Hev b 5.0101, Cyn D, Der p 2, and Rum cr were the only shared allergens across all geographical groups.

Conclusions and Clinical Importance: Microarray profiling can identify specific allergenic components associated with SA in horses, while mathematical modeling of this data can be used for disease classification, highlighting the variability of sensitization profiles between geographical locations and emphasizing the importance of local exposure to the prevalence of different allergens. Frequency scoring analysis can identify important variables that contribute to the classification of SA across different geographical regions.

KEYWORDS
allergen, IgE, microarray, One Health, severe equine asthma

Samuel J. White and Philippe B. Wilson contributed equally as senior authors.

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1 | INTRODUCTION

Severe asthma (SA) in horses is a performance-limiting, debilitating condition which is prevalent in 14% of horses within the Northern Hemisphere.[1] The pathogenesis of this condition remains controversial with many conflicting reports but several studies indicate the role of immunoglobulin E (IgE) through in vitro histamine release assays and allergen-specific IgE (sIgE) analyses of bronchoalveolar lavage fluid (BALF) and sera.[2-4] Specific IgE assays suggest that *Aspergillus fumigatus* (Asp f extract, recombinant (r)Asp f 8, rAsp f 1/a), *A. terreus*, *Alternaria alternata*, *Tyrophagus putrescentiae*, *Saccharopolyspora rectivirgula*, *Eurotium amstelodami*, *Geotrichum candidum*, and *Wallemia sebi* might be implicated in the etiology of SA.[5-10] More recently, 40 further potential allergens of interest were identified from a wide range of genera, including fungi, bacteria, pollen, and arthropods.[11] SA diagnosis is based on clinical history and readily identified clinical signs, which correlate with severity of asthma, furthermore ancillary diagnostic tests such as BALF cytology, lung function testing, hematology, and immunological testing are used to improve diagnostic accuracy.[12-17] While several studies address the potential benefits derived from in vitro allergen assessment in diagnosing SA, commercial applications have historically been hampered due to a lack of statistical approaches for clear disease classification, and the limited range of allergens tested.[18,19] To combat this, a microarray platform has been developed to profile allergen-specific IgE, which enables the serological diagnosis of SA.[20] Moreover, the serological investigation of 138 horses living in varying environments identified that SA is associated with a large sensitization profile, and predominantly involves latex, fungi, mite, and pollen proteins[20]; demonstrating similar profiles to those found with allergic asthma in the human, for which common allergens include *Alternaria alternata*, *A. tenuis*, *Aspergillus fumigatus*, *Dermatophagoides pteronyssinus*, and *D. farinae*.[21] The similarities between equine and human sensitization profiles in asthma is relevant in terms of One Health, in which learning more regarding the health and well-being of one can support knowledge transfer in the other.[22] This microarray platform has potential for the advancement of diagnostics and therapeutics to asthmatic horses.

Despite this, the relationship between geographical location and results of wide-scale allergen profiling has not been explored and is an essential element in establishing the accuracy and appropriateness of protein inclusion for SA microarray-diagnostics. Moreover, the microarray profiling of SA samples demonstrates that an environmentally mixed group of SA horses results in a lower sensitivity and specificity for disease prediction, when compared with environmentally matched samples.[20] It has been hypothesized that allergen-specific serological IgE levels might result from different horse populations and environmental factors.[23] This is further supported by human research in dizygotic and monozygotic twins, which when considered in tandem with genetic factors, demonstrate the substantial influence environmental factors play in specific serum IgE levels.[24] Further work is required to establish the effects of geographical location on specific-IgE in SA-affected horses to enable appropriate diagnostic modeling and future therapeutic development.

This study evaluates the influence of geographical location on wide-scale comprehensive profiling of IgE, and its effects on sensitization profiles and the diagnostic efficacy of microarrays in SEA-affected horses.

2 | MATERIALS AND METHODS

2.1 | Equine samples

A total of 35 sports horses, consisting of environmentally matched samples (ie, each international group housed in the same facility undergoing the same management regime) from Caen, France (5 SEA; 6 control), Indiana, the United States (6 SEA; 6 control) and Quebec, Canada (6 SEA; 6 control) were analyzed. These were modeled to enable reliable comparison of samples with matched controls collected from horses in the same environment, thus accounting for any antigenic stimuli associated IgE responses.

All horses underwent complete physical examination. Specific evaluation of the respiratory system was performed using a validated clinical scoring system.[15] Abdominal lift, nasal flare, cough, tracheal sounds, bronchial tones crackles, and wheezes were assessed when the horse was spontaneously breathing. Clinical scores were assigned by use of a scale (range, 0-25),[15] a SEA crisis was defined as a clinical score ≥12, and control was a clinical score ≤6.[25] Inclusion criteria for the control horses were: no history or physical examination findings[15] of respiratory disease or exercise intolerance, a BALF neutrophil proportion <10%, mast cell proportion <2%, and eosinophil proportion ≤1%,[25] and absence of tracheal mucus.[26] Inclusion criteria for horses with SEA were a history and physical examination findings[15] of respiratory disease, as well as an increased BALF neutrophil proportion ≥25%, mast cell proportion ≥2%, and eosinophil proportion >1% on evaluation of cytology,[25] along with tracheal mucus.[26]

BAL was performed as described,[25] essentially horses were sedated using detomidine hydrochloride (0.01-0.02 mg/kg IV) and butorphanol tartrate (0.01 mg/kg IV). A sterile BAL tube (10 mm outer diameter) was inserted through the nose and wedged into a peripheral bronchus. Local anesthesia was achieved with 60 mL of a 0.4% lidocaine solution during tube insertion. Subsequently, 250 mL of 0.9% NaCl was manually infused and recovered. Manual and automated cell counts were performed on fresh BALF, and cytologic specimens were prepared by cytospin centrifugation and processed with modified Wright stain. Differential cell counts were conducted on a minimum of 400 total cells. Blood was collected from the jugular vein in VACUETTE Serum Clot Activator Tubes, centrifuged at 2000*g* for 10 minutes, serum removed, and stored at −80°C.[20] All experiments were performed in accordance with the relevant guidelines and regulations.

2.2 | IgE sera determination by protein microarray

The comprehensive complex microarray comprised of extracts (n = 153) and pure proteins (n = 231) from a wide range of fungi,

bacteria, pollen, arthropods, and others associated with the equine environment. The extracts, pure and recombinant proteins were obtained from commercial suppliers, and produced in-house, and abbreviated in line with current allergen nomenclature.[27] Fungal and bacterial strains were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen, grown in liquid media, and extracts produced via sonication. Samples were normalized to 0.5 mg/mL protein and printed onto ONCYTE NOVA Nitrocellulose Film Slides

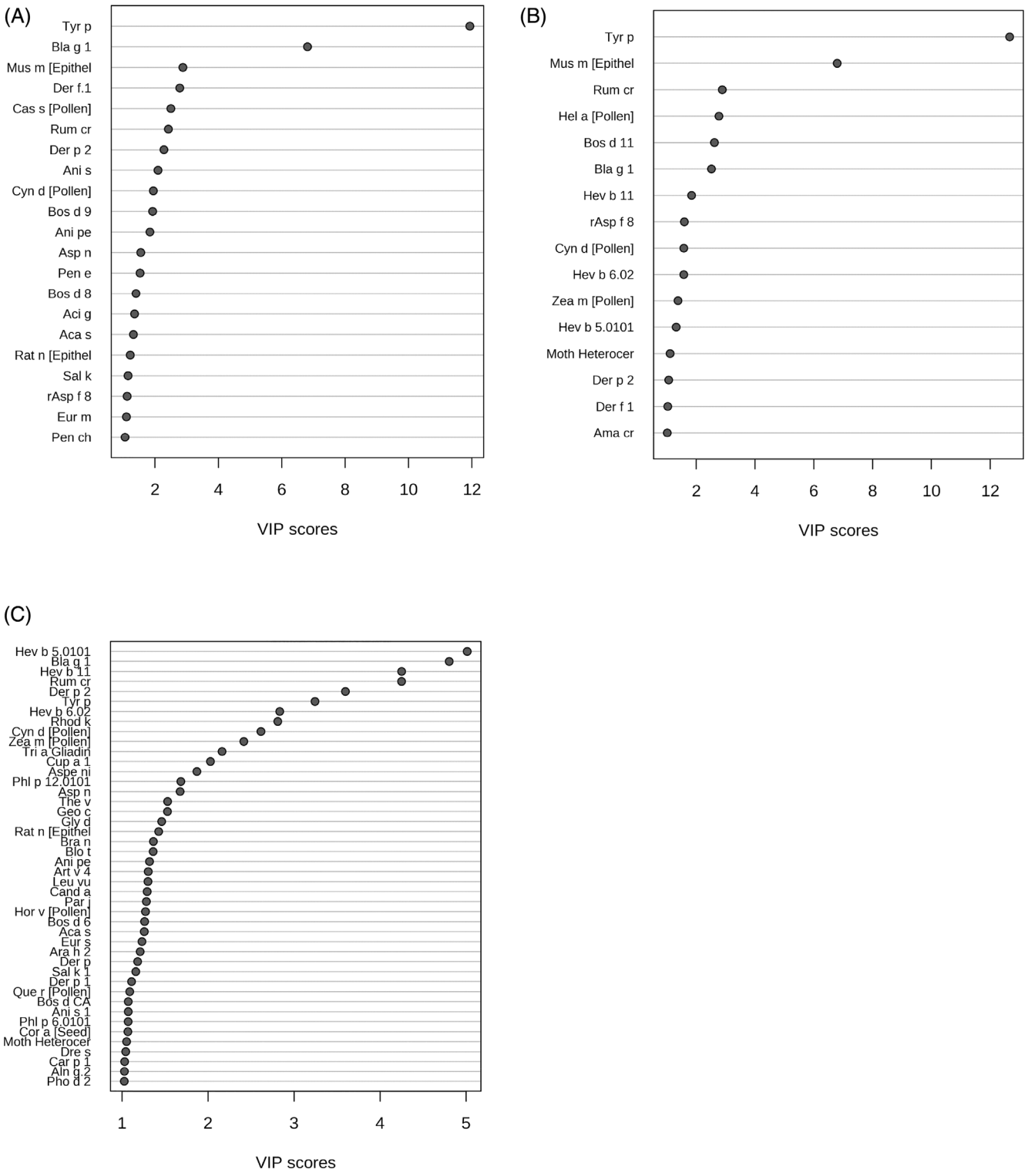

FIGURE 1 Results of statistical analyses for the geographical subsets of horses. (A) Canada; (B) France; and (C) the United States. Variable influence on the projection (VIP) of the proteins included in the microarray calculated by partial least square-discriminant analysis (PLS-DA) after VIP selection. The significance threshold was set to VIP >1. High-low scale refers to IgE quantity based on fluorescent units.

(Grace Bio-Labs, Oregon, USA) using an Ultra Marathon II by Arrayjet, (Roslin, Scotland) to a final spot density of 12 288 spots/slide, with an approximate spot size of 200-µm diameter and replicated twice into 2 blocks on each pad. Slides were analyzed as described by White et al, essentially slides were blocked in BSA solution at 37°C for 3 hours, washed 3 times in PBST (0.05%), followed by 5 washes with Milli-Q water, and dried via centrifugation.[11] Slides were fitted with Proplate slide modules, washed 3 times with PBST (0.2%). Sera samples were diluted 1:2 with 4% BSA in 0.4% PBST, and added to each well, excluding well 4, which was a control. The Proplate was fitted with an adhesive seal strip and incubated for 16 hours at 4°C, washed 3 times with PBST (0.05%), incubated for 2 hours at 37°C in a ThermoHybaid (HyPro 20) with anti-horse IgE (BioRad, #MCA5982GA) in 1% BSA in 0.2% PBST. They were washed a further 3 times with PBST (0.05%) and incubated for 1 hour at 37°C in the ThermoHybaid with DyLight 649 conjugated anti-mouse IgG (Rockland, Product #610-443-040) in 1% BSA in 0.2% PBST. Slides were then washed 3 times with PBST (0.05%) followed by 3 washes with Milli-Q water and dried via centrifugation.[11]

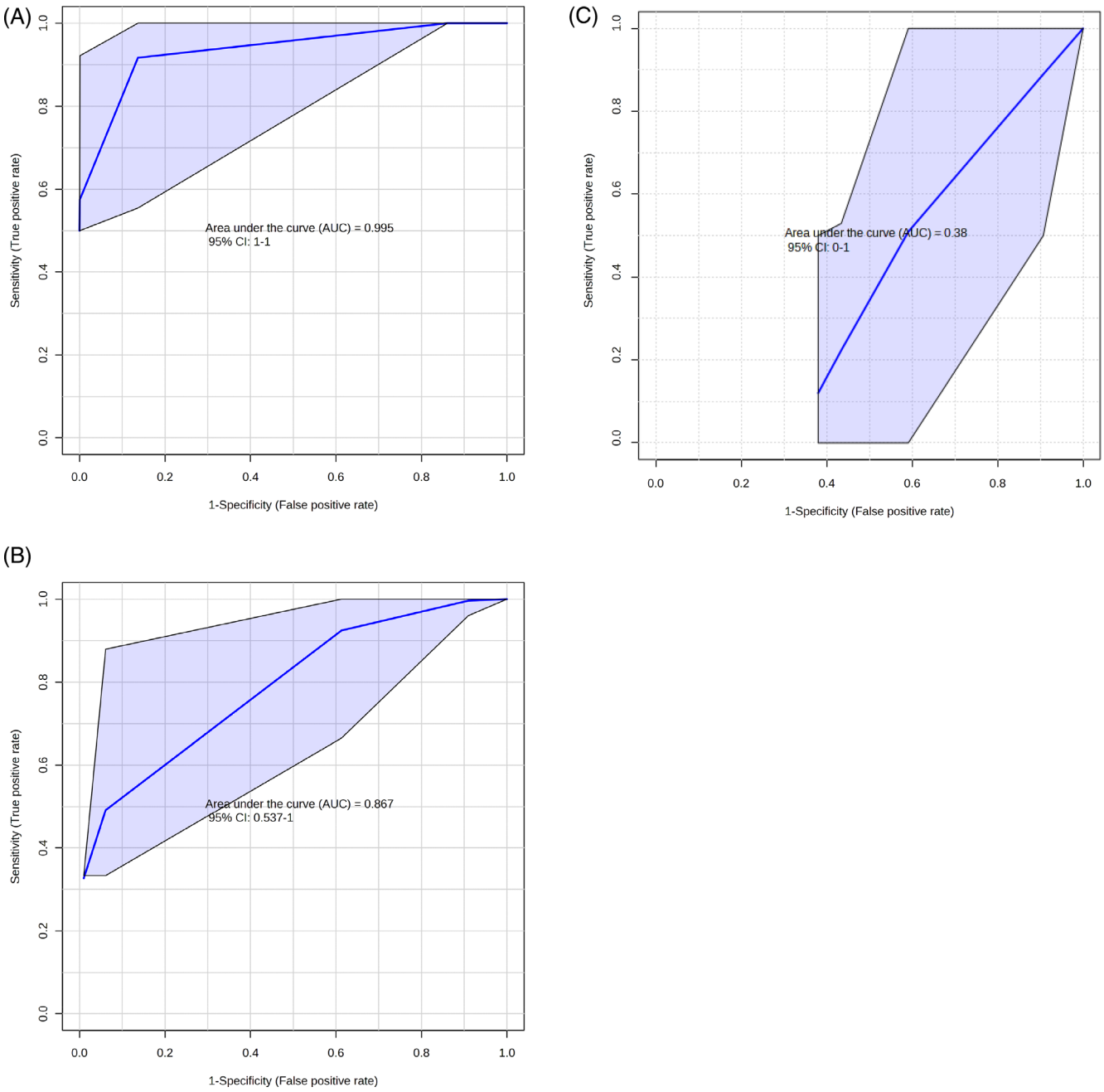

FIGURE 2 Receiver operating characteristics (ROC) curve based on the cross-validation performance with a 95% confidence interval computed for the PLS-DA model. (A) Canada; (B) France; and (C) the United States.

2.3 | Data analysis

Processed slides were scanned in a GenePix 4000B (Molecular Devices, USA) with the PMT settings 440 and 310 at 635 and 532 nm and saved as TIF files. Images were processed in GenePix Pro software v6.0.1.27 (Axon Instruments) and saved as comma-delimited text files. 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 serum, the results of which were subtracted from all other pads to account for any auto-fluorescence or non-specific binding. Clinically healthy horses were used as control.

An R-based software was used to carry out mathematical modeling. Principal Component Analysis was performed, before Partial Least Squares Discriminant Analysis (PLS-DA) to assess separation of predefined classes of samples (ie, affected/non-affected horses) for each geographical location.[20] 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. This is a measure of the contribution that a specific variable has on the model. In an effort to reduce the background noise and improve robustness of the mathematical model, a second iteration of modeling was conducted using the main VIPs identified in the calibration step. The predictive performance of the model was evaluated, via cross-validation, including Q2 (an estimate of the predictive ability of the model). The prediction error is then summed over all samples (Predicted Residual Sum of Squares or PRESS), the PRESS was then divided by the initial sum of squares and subtracted from 1 to normalize to R2. For each model, 1000 permutations were performed. The receiver operating characteristic (ROC) curve was generated by Monte-Carlo cross-validation using balanced sub-sampling. For each Monte-Carlo cross-validation, 2-thirds of the samples were used to evaluate the feature importance. The area under the ROC curve (AUC) and the prediction (Prediction accuracy during training with 1000 permutations) matrix were obtained to assess sensitivity and the specificity.[28] A 95% confidence interval was used for the models. Frequency analysis was conducted on VIP scores across all geographical groups to establish common proteins important for class division.

3 | RESULTS

The PLS-DA classification methods using the geographical subsets of environmentally matched samples (n = 35) demonstrating the varied nature of sensitization profiles geographically through VIP scores (Figure 1), as well as the variable predictivity of the PLS-DA models (Figure 2).

The ROC curves generated for the PLS-DA models in each region indicated the balance between sensitivity (the proportion of true positives correctly identified) and specificity (the proportion of true negatives correctly identified) at different thresholds. The area under the ROC curve (AUC) is a measure of the overall performance of the model, with a value of 1 indicating perfect discrimination and a value of 0.5 indicating no discrimination beyond chance. The results of the study showed that the PLS-DA models had a high discriminatory power in predicting SEA in horses from Canada (AUC = 0.995) and France (AUC = 0.867). In contrast, the AUC value for horses from the United States was much lower (AUC = 0.380), indicating poor discriminatory power.

Frequency scoring showed the only shared allergen associated with classification across all geographical groups were, Hev b 5.0101, Cyn D, Der p 2 and Rum cr (see Figure S1).

4 | DISCUSSION

In this international study of horses with SEA, we discovered significant regional variations in IgE-sensitization profiles, influenced by environmental factors, emphasizing the importance of local allergen exposure. Furthermore, our mathematical modeling for disease classification established varying discriminatory power among regions, underlining the need for region-specific allergen identification. Additionally, common allergens shared across regions were identified, highlighting their potential significance in precision medicine for SEA diagnosis and treatment. Microarrays have been used for the analysis of IgE,[18,19] and the identification of various allergen components linked to asthma,[11,20,28] while also demonstrating the reliability of IgE microarray analysis when compared with standard laboratory methods such as ELISA, UniCAP, and immunoblot tests.[20,29] Mathematical modeling of profiling data for disease classification and allergen identification is a widely adopted technique in human allergy research, yet its application in the veterinary sector has been limited.[30] Based on these principles, we utilized state-of-the-art technological developments and mathematical modeling to explore the geographical influences on SEA.

The VIP outputs demonstrate the variability of sensitization profiles between geographical locations, emphasizing the importance of local exposure to the prevalence of different allergens. These variations are likely dependent on a number of factors, including the climate, vegetation, and other environmental factors in a particular location, meaning horses residing in different regions might be more or less likely to be sensitized to certain allergens.[31,32] Variability in AUC values highlighted the potential limitations in singular mathematical modeling as an auxiliary diagnostic technique globally, and rather highlights the importance of assessing individual sensitization profiles on a case-by-case basis for clinical recommendations. Frequency scoring highlighted a range of common allergen associated between all geographical locations, all of which were previously listed as allergen relevant for class division in SEA mathematically modeled geographically mixed groups.[20] This could indicate, that with wider geographical assessment global allergen commonality could be established to a level by which mathematical modeling will enable full, as oppose to auxiliary, diagnosis.

The PLS-DA models had a high discriminatory power in predicting SEA in horses from Canada and France. In contrast, the AUC value for horses from the USA was much lower, indicating poor discriminatory

power. This suggests that there might be differences in the allergen profiles associated with SEA in horses from different regions, and that these differences might affect the performance of allergen profiling as a diagnostic tool when using mathematical modeling for disease classification.

The sensitization profiles revealed significant regional variations in specific IgE among different cohorts. These results align with the idea that environmental factors significantly influence specific IgE levels in horses. In this context, significant variations are observed in specific IgE levels when testing a limited range of allergens among different stud farms, which can be attributed to environmental factors.[23] Overall, these findings highlight the complex interplay between genetics and environment in shaping IgE levels in horses. Studies conducted on population-based cohorts in humans from individual countries, such as the UK, Germany, and Italy have validated observations on molecular sensitization patterns in these countries.[33,34] In our study, we observe regional variations in the exposome, particularly related to climate, with marked discrepancies in temperature between the United States, Canada, and France. These differences might contribute to higher rates of pollen sensitization among individuals in the United States, consistent with prior research showing that elevated temperatures influence pollen levels, potentially leading to increased pollen sensitization.[35] The higher prevalence of pollen sensitization in the USA group might be attributed to significant cross-reactivity with group 1 allergens found in various grass species. Group 1 allergens, unlike other groups of grass pollen allergens, are ubiquitous across all grass species, resulting in a higher overall prevalence of sensitization to grasses.[36] For tree pollen allergens, significant differences in sensitization profiles were noted, which clearly reflect the different tree exposomes in these regions. Cas s was the prominent tree pollen associated with class division within the Canadian group, whereas Que r was only seen within the American group. The presence of dust mite allergens, both Der p and Der f, depends on humidity,[37] which has been reflected in geographical sensitization profiles in the human.[32] Interestingly, Der p and Der f were consistently significant for class separation between all groups, this could be because of the relative humidity of the geographical sampling areas, as well as partially because of the relative humidity of the stabling environment, particularly in combination with moisture content of forage and bedding and time of baling.[38-40] Unlike house dust mites, the fungus *Alternaria alternata*, has shown to be an outdoor allergen, although often found contaminating the indoor environment, and growing best in a warm climate.[41] Alt a and Alt a 1 have previously been associated with SEA-affected horses,[11,20] however was not significant for any of the group predictions in this study.

The frequency scoring analysis identified 4 VIPs that were shared across all 3 regions and significantly contributed to the classification of SEA-affected horses. The 4 shared VIPs were identified as Hev b 5.0101, Cyn D, Der p 2, and Rum cr. These VIPs are all known allergens associated with respiratory allergies in horses.[11] Hev b 5.0101 is a major latex allergen that causes asthma symptoms in individuals sensitized to latex in humans, and is one of the most notable allergens associated with classification in SEA.[20] Cyn D is a major allergen from Bermuda grass pollen that has been implicated in allergic rhinitis as well as asthma in humans and horses.[20,42] Der p 2 is a major allergen from dust mite, and is a significant risk factor for asthma in individuals sensitized to dust mite allergens, as found in horses.[6] Rum cr is a major allergen from the common curled dock, which is a common cause of allergic rhinitis and asthma in individuals exposed to pollen.[43] These allergens are important contributors to the pathogenesis of asthma in various studies, and their detection in equine asthma samples across different geographic regions highlights their potential role in the development of the disease. The fact that these VIPs were shared across all 3 regions suggests that they might be important drivers of SEA globally. Overall, these findings highlight the potential utility of frequency scoring analysis in identifying important VIPs that are shared across different geographical regions and might play a key role in disease etiology. Similarly, rAsp f 8, which was previously identified as one of the most significant allergen for classification of SEA,[20] was only significant in the Canadian and French group, further emphasizing the paramount nature in further understanding exposome interactions.

Most notably, we have detected diverse molecular IgE sensitization profiles in different regions globally. This has important implications for precision medicine approaches, such as allergen avoidance, and the precise prescription of allergen-specific immunotherapy.[18,44] Molecular diagnosis is particularly critical in identifying the actual sensitizing allergen sources that may be concealed by cross-reactivity when using allergen extracts.[36,45] This emphasizes the need for accurate identification and avoidance of allergens in precision medicine approaches. With increased use of the horse as a model for human asthma, this approach has substantial implications within the One Health field.[46]

In conclusion, this study has provided valuable insights into the role of environmental factors in equine asthma and highlighted the importance of accounting for these factors in subsequent studies on serum IgE levels. The findings of this study suggest that allergen-specific IgE titers can be influenced by a range of environmental factors, and further investigations are needed to fully understand the complex interplay between diagnosis, environment, and allergen-specific IgE levels in horses. The results of this study could inform the development of more targeted and effective approaches to diagnosing, treating and managing equine asthma, ultimately leading to improved health outcomes for affected horses.

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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 Nottingham Trent University, ARE897.

HUMAN ETHICS APPROVAL DECLARATION

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

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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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