**Development of a Comprehensive Protein Microarray for Immunoglobulin E Profiling in Horses with Severe Equine Asthma (sEA)**

**Abstract:**

**Background -** Severe equine asthma (sEA) is a prevalent, performance-limiting disease associated with increased allergen-specific immunoglobulin E (IgE) against a range of environmental aeroallergen.

**Objective –** To develop a protein microarray platform to profile IgE against a range of proven and novel environmental proteins in sEA-affected horses.

**Animals -** Six sEA-affected and six clinically healthy warmblood performance horses.

**Methods -** A protein microarray (n=384) was developed using protein extracts and purified proteins from a large number of families including pollen, bacteria, fungi and arthropods associated with the equine environment. Conditions were optimized and assessed for printing, incubation, immunolabeling, biological fluid source, concentration techniques, reproducibility and specificity.

**Results –** This method identified a number of novel allergens, while also revealing an association between sEA and pollen sensitization. Immunolabeling methods confirmed the accuracy of a commercially available mouse anti-horse IgE 3H10 source (R2=.91). Biological fluid source evaluation demonstrated that sera and bronchoalveolar lavage fluid (BALF) yielded the same specific IgE profile (average R2=.75). Amicon centrifugal filters were found to be the most efficient technique for concentrating BALF for IgE analysis at 40-fold. Overnight incubation maintained the same sensitization profile while increasing sensitivity. Reproducibility was demonstrated (R2=.97), as was specificity via protein inhibition assays. As expected, arthropods, fungi and pollens have shown the greatest discrimination for sEA.

**Conclusion -** Here we have established that protein microarrays can be used for large scale IgE mapping of allergens associated with the equine environment. This technology provides a sound platform for specific diagnosis, management and treatment of sEA.

1. **Introduction**

Severe equine asthma (sEA) is a performance limiting, allergic response to inhaled allergen in genetically predisposed horses, that effects approximately 14% of the UK equine population 1–3. Allergen exposure in affected horses results in small airway inflammation, mucus hypersecretion and bronchoconstriction; altering pulmonary resistance, dynamic compliance and pleural pressure 4–6. The predominant source of these aeroallergens is the organic dust portion of forage and bedding, which contains fungi, bacteria, pollen and arthropods 7–11. Removal from the aeroallergen-rich stabling environment results in a level of remission 12, but owner compliance is limited due to seasonality, competition schedule, health issues and nutritional demands. Treatment with corticosteroids and bronchodilators provide short-term relief; however, such therapeutic approaches have been associated with undesirable adverse effects and their use is prohibited under *Fédération Équestre Internationale* and jockey-club rules 13,14. Allergen avoidance is the cornerstone to prevention and effective treatment, however the efficacy of the latter approach relies on identification of causal allergens 15. Currently, the major obstacles to diagnostic and therapeutic developments include a) major limitations in the number of allergens screened to establish causal agents, and b) the lack of a clinically applicable *in vitro* test.

The pathogenesis of this condition remains unclear; however, several studies have demonstrated the involvement IgE immunoglobulin E (IgE) through *in vitro* histamine release assays 16–18, natural hay and straw challenges 19, intradermal testing 20 and specific IgE (sIgE) analysis of bronchoalveolar lavage fluid (BALF) and sera 21,22. sIgE assays suggest that *Aspergillus fumigatus, Alternaria alternate, rAsp f 8*, *Tyrophagus putrescentiae*, *Saccharopolyspora rectivirgula,* Asp f 1/a, *Aspergillus terreus, Eurotium amstelodami, Geotrichum candidum* and *Wallemia sebi* are implicated in the etiology of sEA 8,21,23–25. Although a vast number of recombinant proteins are available 26, advancements in causal allergen identification has been limited due to the viability of testing with classic methods, such as ELISA, which are time-consuming, expensive, and require large quantities of samples and reagents 27.

In recent years, protein microarrays have been gaining popularity in allergy diagnostics due to their ability to assess the interaction of thousands of proteins with specific immunoglobulin isotypes using techniques such as fluorescence, on a miniaturized scale; a technique known as microarray profiling 28. This circumvents the aforementioned limitations associated with techniques, such as ELISA, enabling multi-allergen testing to assess complex sensitization profiles. Furthermore, with specific allergen these tests show similar sensitivity to standard laboratory methods, including ELISA, UniCAP, CAP/RAST, ImmunoCAP and immunoblot test 29–31. Previously published sensitivity and specificity values using protein microarrays have demonstrated the high discriminatory power of the protein extracts and pure recombinant *Culicoides* proteins associated with insect bite hypersensitivity in the horse 22.

The aim of the presented study was to develop, and demonstrate, that widespread allergen profiling using microarray methods enables fast and accurate IgE profiling of sEA. Furthermore, we wanted to analyze the correlation between BALF and sera specific IgE profiles, a crucial consideration with respect to diagnostic sample requirements. Profiling data allows for diagnostic and therapeutic advancements.

**2.0 Materials and methods**

**2.1 BALF and sera samples**

Clinical assessment including physical examination, pulmonary function tests and BALF cytology was used to define the inclusion and exclusion criteria for selection of six horses with sEA and six control horses 5. BALF was collected as previously described 32, filtered through a 100 ml syringe filter (Biocomma, Shenzhen, China), and decanted into 10 ml aliquots in 15 ml centrifuge tubes with the addition of Thermo Scientific Pierce Mini-Protease Inhibitor Tablets – EDTA free (product # 13437766). The mixture was gently agitated and incubated at 4oC for 10 min prior to the addition of 2.5 ml glycerol (Fisher Scientific, Leicestershire, UK) and stored at -80oC until analysis. To concentrate, BALF was thawed, maintained at 4oC and filtered with a Sartorius Stedium 0.45 μm filter syringe (product # 17598). BALF samples were then concentrated in an Amicon Ultra-15 centrifugal filter (product # UFC905024) and used immediately. Blood was collected and sera prepared as previously described 33, prior to storage at -80oC until analysis, at which time the samples were thawed at room temperature and placed on ice.

**2.2 Proteins, printing and hybridization**

In order to maximize utility, the microarray was designed to be as comprehensive as possible by containing extracts and pure proteins from a wide range of protein families derived from predominantly, fungi, bacteria, pollen and arthropods. The extracts and pure proteins were obtained from commercial suppliers, produced in-house and from donations. Due to the limited commercial availability of some bacterial and fungal protein extracts, it was necessary to produce them in-house. Lyophilized purified samples of the desired strain were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (<https://www.dsmz.de>) and grown in 150 ml of liquid media according to the supplier’s recommendation (250 ml Erlenmeyer flask). Once grown, the media were centrifuged in 50 ml tubes at 4000 × g for 10 min, supernatant was carefully removed before washing the individual pellets in 5 ml of PBS. The tubes were centrifuged at 4000 × g for 10 min, supernatant removed and 1 ml of lysis buffer solution added to each tube (PBS, 0.5% TritonX-100 w/v and Thermo Scientific EDTA-free protease inhibitor mini-tablet). The re-suspended pellets were pooled into a single 50 ml centrifuge tube, placed on ice, and sonicated using an MSE Soniprep 150 (15 seconds sonication with 30-second cooling periods in-between for 10 cycles). Subsequently, the solution was filtered through a Nalgene 0.45 um syringe filter (product # 190-25-45) and protein content quantified using a Pierce BCA Protein Assay Kit (product # 23225). The remaining solution underwent lyophilization, was re-suspend in MiliQ water with 10% glycerol (filtered through a 0.02 um syringe filter) and normalized to 1mg/ml protein and stored at -80 oC.

Bronchoalveolar lavage concentration work was initially conducted using slides described in Marti et al., 2015 consisting of extracts (n=240) and pure proteins (n=120) from a range of protein families including amongst others fungi, pollen and arthropod to establish the optimal BALF concentration to be utilized in subsequent development work. In-house extracts not present in the initial array setup were initially trialed by printing normalized samples (1 mg/ml protein) onto 16 pad FAST™ slides (Whatman Schleicher & Schuell, Dassel, Germany) using a QArraylite arrayer (Genetix, UK). After sample selection, a new set of 384 proteins (supplementary data) were printed in a professional setting using a Marathon microarrayer (ArrayJet, Roslin, Scotland) printer essentially as described in Marti et al., (2015) with an approximate spot size of 200 μm diameter and replicated with even spacing two times across each of the individual 16 pads into two identical blocks to final spot density of 12,288 spots/slide. For alignment and quality control, spots of Cy3, Cy5 and PBS were printed onto each slide. Once printed, slides were blocked for 3 h at 37 °C in 3% BSA (w/v) in PBS inside a Corning 5 slide holder (product # 40082) using a mini hybridization oven (Appligene, USA), washed three times for 2 min in PBS containing 0.05% (w/v) Tween-20, followed by five times 1 min washes with MiliQ water, and dried by centrifugation (MSE Mistral 3000i, Sanyo, UK) at 1000 × g for 10 min at room temperature.

Slides were fitted with Proplate slide modules (Grace Bio-Labs, product # 204862) and washed three times (60 second dwell time) with PBST (0.2 %). Samples (BALF / sera) were diluted 1:2 with 4 % BSA in PBST (Tween at 0.4% w/v) containing Thermo Scientific Pierce Mini-Protease Inhibitor Tablets – EDTA free (product # 13437766)(1 tablet in 5 ml), which has previously been passed through a Whatman 13 mm, 0.45 μm filter syringe (product # 6784-1304). 100 μl of prepared sample was added to each well, excluding well 4, which was used as control and filled with 100 μl of the dilution solution (1:2) in PBS. The Proplate was fitted with an adhesive seal strip and incubated for 16 h at 4oC on the Stuart mini see-saw rocker (SSM4) at 13 oscillations / minute. Slides were washed three times with PBST (Tween at 0.05%) using the BioTek plate washer and incubated for 2 h at 37oC in a ThermoHybaid (HyPro 20) at AVS 3 with 100 μl per well of mouse anti-horse IgE (BioRad, product # MCA5982GA) 1:400 in 1% BSA in PBST (Tween at 0.2% w/v), washed a further 3 times with PBST (Tween at 0.05%) and incubated for 1h at 37°C in the ThermoHybaid with 100 μl per well of DyLight 649 conjugated anti-mouse IgG1 (Rockland, product # 610-443-040) 1:400 in 1% BSA in PBST (Tween at 0.2% w/v). The slide was then washed 3 times in PBST (Tween at 0.05%) followed by three washes with Milli-Q water, and dried via centrifugation at 300 × g for 10 mins (Mistral 3000i, rotor 43124-708).

**2.9 Data analysis**

Processed slides were scanned in a GenePix 4000B (Molecular devices, USA) with the photomultiplier tube (PMT) settings at 440 and 310 at 635 and 532 nm respectively 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. Fluorescence values were calculated for each spot by subtracting local background from the median fluorescence value of the spot. One pad per slide contained all reagents with addition of PBS instead of sera for control purposes, these results were deducted from samples on the same slide to account for any protein autofluorescence and non-specific binding. Further analysis and data presentation were carried out using Microsoft Excel. Average fluorescence values for each protein were compared between sEA and control groups using a conventional Z-test in Excel (Microsoft, USA). The Benjamini-Hochberg method was used to account for false discovery at a rate of .05. Benjamini-Hochberg corrected values were considered significant at *P <* .05. Linear regression (coefficient of determination) of IgE fluorescence values for all proteins (n=384) was used to establish the relationship between BALF and sera, reproducibility of results and varying mouse anti-horse IgE sources. Bronchoalveolar lavage fluid concentration techniques and concentrations were tested by one-way ANOVA. Tukey’s HSD test for multiple comparisons was run if significant differences were found (*P <* .05).

**3.0 Results**

**3.1 Optimizing sera incubation conditions**

**3.1.1 BALF concentration techniques**

BALF concentration employing Amicon and PD10/lyophilizing methods were compared using a BALF pool from six horses (n = 3 sEA and 3 control). Total IgE for each protein group was used to compare concentration methods (figure 1A), revealing no significant difference (p<0.05) between concentration techniques. The Amicon concentration method was used to evaluate optimal BALF concentration via total IgE fluorescence for each protein group, revealing that a plateau was reached at 40-fold concentration (figure 1B). Therefore, all subsequent BALF concentrations were carried out via Amicon filtration to a 40-fold final concentration.

**3.1.2 Incubation time**

Two conditions were tested for optimal sera incubation times using a sera pool from six horses (n = 3 sEA and 3 control); 3 h at 37oC as previously used for equine sera 22 and overnight (16 h) at 4oC which has previously been shown to be more sensitive in human studies 34. As shown in figure 2, the IgE profile between the two incubation times significantly correlated (R2 = .76), however, when the sera was incubated for 16 h at 4oC, it was more sensitive with 28.08% of proteins showing positive reactions, compared to the 4 h incubation, which showed 16.44% of proteins with positive reactions (data not shown). *Ergo*, subsequent serological incubations were conducted overnight at 4°C to increase sensitivity.

**3.1.3 Comparison of specific IgE in BALF and sera**

Bronchoalveolar lavage samples concentrated by Amicon (40-fold) were compared with sera from six horses (n = 3 sEA; n = 3 control) and correlation coefficients calculated for each separate protein group. Strong correlations were demonstrated between BALF and sera (table 1), ergo, all subsequent incubations were conducted with sera as it is far easier to obtain, is less invasive, more economical and stable to transport. Horse 5, a clinically healthy horse, showed poor BALF / sera correlations across all protein groups, which was thought to be a result of the horse recently changing to a different barn on the same yard, and therefore localized IgE production in the lung due to environmental allergen correlated poorly with serological IgE 23,35.

**3.2 Reproducibility**

**3.2.1 Printing lot variation**

The effect of printing lot on reproducibility of the protein microarray was assessed using two microarray slides printed on the same day. Sera from three sEA and three control horses was hybridized on the two slides simultaneously. Fluorescence values from replicate arrays were evaluated using linear regression and showed R2=.97, demonstrating the fluorescence values from the array were repeatable between printing lots (data not shown).

**3.2.2 Comparison between monoclonal mouse anti-horse 3H10 sources**

Two mouse anti-horse IgE monoclonal antibodies (derived from the 3H10 clone) were compared via linear regression of the fluorescence values using a sera pool from six horses (n = 3 sEA; n = 3 control), this included the original 3H10 from Wilson et al., (2006) 36 and the commercially available BioRad 3H10 (product # MCA5982GA).

As shown in figure 3, the fluorescence values from the array showed a correlation coefficient of R2=.91, demonstrating a significantly similar IgE profile.

**3.3 Specificity – protein inhibition assay**

In order to test the IgE specificity of IgE-protein binding, a protein inhibition assay was performed to assess cross-reactivity, in which pooled sera was spiked with several proteins in serial dilution and its effect on related and neighboring proteins evaluated. A protein inhibition assay enables the confirmation of specificity of an antibody against the target protein, and is usually conducted with several proteins to confirm both the antibodies specificity to the target protein and assess potential cross-reactivity 34. Two different protein inhibition groups were used, each containing two different proteins. Group 1 consisted of *Blattella germanica* (Bla g 1) and *Rumex crispus* (Rum cr), and group 2 consisted of *Penicillium notatum* (Pen ch) and *Acinetobacter gerneri* (Aci g). Interestingly, reduced fluorescence from proteins other than those targeted was also observed, indicating either non-specific inhibition or some similarity between the allergenic components of the proteins. Group 1 inhibition group showed no non-specific binding in the bacteria, arthropod or fungi groups, however, cross-reactivity was seen among grass pollens – most notably *Cynodon dactylon*, *Rumex crispus*, *Zea mays* and *Anthoxanthum odoratum*. Group 2 inhibition group demonstrated no non-specific binding in the bacteria, arthropod and pollen groups, however, cross-reactivity was seen between *Aspergillus niger, A. versicolor, Penicillium expansum, P. notatum, A. nidulans* and *A. fumigatus* (figure 4).

**3.4 Allergen comparison**

Whether the prototype array was able to identify novel allergens associated with sEA was assessed using Z-tests with Benjamini-Hochberg adjusted p-values between six sEA and six control sera samples. The results shown in table 2 confirmed the ability to conduct IgE profiling and identify potential sEA allergens via microarray methods. As expected, it confirmed the presence of fungi and mite as main reactants in the sEA population, while also identifying an association with pollen which has not previously been implicated.

**4.0 Discussion**

We have previously demonstrated the sensitivity and specificity of microarrays in the diagnosis of insect bite hypersensitivity 22, confirming the high discriminatory power of complex extracts and pure recombinant *Culicoides* proteinsassociated with the allergy. Based on these principles, an array was constructed to enable multi-allergen testing and assess the complex sensitization of profiles associated with sEA in a single assay, based on equine environmental proteins. The use of protein extracts was essential as so few proteins have been assessed in relation to sEA to date; therefore, in the early stages of development it was wise to use a range of extracts to maximize coverage, in combination with pure proteins, where available or where the allergen has been previously associated, thus simultaneously maintaining specificity. Furthermore, we observed comparable accuracy between natural extracts and recombinant allergens, whereas some authors suggested the use of the recombinant component alone may be insufficient for some allergens 37. Although the eventual goal will be to move towards component resolved diagnostics (CRD) utilizing individual allergen molecules for increased sensitivity and minimizing cross-reactivity, the genus/species must be identified to enable the production of pure proteins. CRD offers vastly increased accuracy over routine diagnostic tests (skin prick and specific IgE determination) 38, and enables the accurate selection of allergens to be used for allergen immunotherapy 39. Moreover, the identification of sensitization to pure proteins will assist in diagnostic and therapeutic advancement. This microarray approach has several advantages: allows substantial allergen profiling with minimal sample, collection of less invasive readily obtained *in vivo* samples, permits automation and enables the generation of mathematical predictive models to assist in clinical allergy diagnosis.

Protein microarray methods primarily consist of two steps: firstly, the printing of proteins onto the nitrocellulose slides, and secondly the profiling of equine IgE. Printing methods are well established; therefore, the latter was optimized to enable analysis of sEA BALF and sera samples. Although it has been suggested that developing technology means sensitivity is such that many immunoglobulin isotypes in BALF can be assessed un-concentrated to the nanogram or microgram, it is often not possible to detect allergen-specific IgE in BALF due to the low concentration of this isotype present 23,40. Therefore, concentration techniques must often be employed to assess BALF IgE. Lyophilization 41 and centrifugal filtration methods 42,43 have been successfully utilized to concentrate BALF for immunoglobulin analysis, however certain techniques such as ammonium sulphate precipitation, can result in denaturation of the liable epitope(s) 44. Similarly, lyophilizing BALF samples without desalting results in high levels of sodium chloride, which can denature proteins. The most commonly utilized BALF concentration technique is the centrifugal filter (Amicon Ultra-15 or Centricon-10), to a 10-fold concentration of immunoglobulin analysis 42,45–49, however, even this technique results in a 20% loss of specific IgE and IgG 46. On collection of BALF for immunoglobulin analysis protease inhibitors must be added to prevent proteolytic cleavage of proteins, which would otherwise leave the immunoglobulin unviable. Similarly, immunoglobulin in BALF is greatly affected by freeze-thaw cycles, so the inclusion of a cryopreservative in the form of glycerol has been shown to be effective 50. Therefore, glycerol was added as a cryoprotectant to a final concentration of 20% to help stabilize the proteins and prevent formation of ice crystals during freezing that destroy protein structure. Unfortunately, samples containing cryopreservative tend to thaw during lyophilization, meaning it was not possible to achieve a 40-fold concentration using this technique. To avoid this and remove sodium chloride concentration, samples underwent a buffer exchange using a PD-10 column prior to lyophilization. Bronchoalveolar lavage concentration levels and techniques were trialed, varying hybridization temperatures and times were assessed, and BALF / sera analyzed. The greatest binding capacity was observed with overnight incubation at 4oC. When using BALF the greatest binding was seen using the Amicon filter and the PD-10 elution column/lyophilizing at a 40-fold final concentration. The Amicon filter was quicker, easier, reduced risk of contamination and had previously been utilized, therefore this technique was selected. Interestingly, during the optimization a biased response toward pollens was observed. Plants are polyploids and show a great number of gene duplications, hence high cross-reactivity between species are generally observed. It is our experience that pollen response in humans and other animals are commonly amplified 51. Due to the significant correlation of BALF and sera (average R2=.75), sera was used in subsequent analysis due to ease of use. Previous work comparing the specific IgE profile of BALF and sera have utilized ELISA techniques, has been limited, and contradictorily to date. The authors concluded that although BALF may be valuable for analysis, sera was of little clinical relevance 21,23. Here we demonstrated the ability to profile unconcentrated sera instead of BALF to assess potential allergens. This has several advantages as sera is far easier to collect, store and prepare for analysis in comparison to BALF. Collection is also less stressful for the horse. Moreover, it holds further potential in the use of diagnostic microarrays as sera is far more stable to ship for analysis 52.

Repeatability is an essential factor in the development of new diagnostic tests. *Ergo*, the effect of printing lot and Mouse anti-horse IgE 3H10 sources were assessed. These results confirmed the reproducibility between printing lots (average R2=.97). The original Mouse anti-horse IgE 3H10 used was that from the Wilson et al., (2006) study. The commercial availability of antibodies is essential in diagnostic tests, therefore the Wilson et al., (2006) 3H10 clone was compared with the commercially available BioRad 3H10 (product # MCA5982GA), confirming reproducibility with the commercially available clone (R2=.90).

Specificity is an important aspect of protein microarrays, which was confirmed by a protein inhibition assay. In this assay some cross-reactivity was seen, predominantly with grass pollens, as well as, *Aspergillus* and *Penicillium*. Sridhara et al.,(1995) reported pollens from grasses (Poaceae) often show high immunological cross-reactivity, potentially indicating common antigenic / allergenic component(s) 53. Furthermore, cross-reactivity was identified between the genus *Penicillium* and *Aspergillus*, which is expected as taxonomically, the genus *Penicillium* and *Aspergillus* have many similarities, as both produce and contain galactomannans with similar galactofuranosyl and immunogenic side-chains. It is worth noting cross-reactivity in the fungi group was only seen with whole protein extracts, emphasizing the importance of including pure proteins. Analysis of human sera in a variety of assays has indicated that *Aspergillus fumigatus* contains determinants in common with *Cladosporium*, *Candida, Alternaria*, *Trichophyton* and *Epidermophyton* 54*,* however cross-reactivity was not identified on this occasion.

Several allergens of interest identified here were consistent with those previously identified as sEA-associated through ELISA, western blot and RAST methods (*Aspergillus fumigatus, Alternaria alternate, Eurotium amstelodami,* and *Geotrichum candidum*). This was the largest panel of proteins tested with a controlled sEA group to date, and thus revealed new and relevant allergens. Several sEA-associated allergens identified in this study have previously been associated with allergic asthma in the human (*Dermatophagoides farinae; Blattella germanica; Aspergillus* *restrictus; Dermatophagoides pteronyssinus*). The novel sEA-associated allergen identified in this study strongly implicate fungi and mite as the main reactants, as well as, revealing a previously unidentified reaction with pollens. This confirms the future potential of specific IgE as a biomarker for the serological diagnosis of sEA.

The results of this study have clearly established a reliable protein microarray for large scale IgE profiling of equine environmental proteins, confirming identified sEA-associated allergens and elucidating a range of previously unidentified allergens. The technique is sufficiently sensitive and specific to differentiate between sensitized allergens in sEA and control horses. Furthermore, the developed serological assay enables accurate identification of an individual horse’s sensitization profile. This information provides a reliable, fast and repeatable method for screening a wide variety of potential allergens found in the stable environment in a miniaturized and affordable format, while offering a platform to support management and treatment of this debilitating respiratory disorder in horses.

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