Veterinary Immunology and Immunopathology 167 (2015) 171–177

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Novel *in vitro* diagnosis of equine allergies using a protein array and mathematical modelling approach: A proof of concept using insect bite hypersensitivity

E. Marti[b,*], X. Wang[a], N.N. Jambari[a], C. Rhyner[c], J. Olzhausen[c], J.J. Pérez-Barea[a], G.P. Figueredo[d], M.J.C. Alcocer[a]

[a] School of Biosciences, University of Nottingham, Sutton Bonington campus, Loughborough LE12 5RD, UK
[b] Department of Clinical Research and Veterinary Public Health, University of Bern, Switzerland
[c] Swiss Institute of Allergy and Asthma Research, University of Zürich, Davos, Switzerland
[d] School of Computer Sciences, Advance Data Analysis Centre (ADAC), University of Nottingham, Jubilee Campus, Nottingham, UK

ARTICLE INFO

Article history:
Received 17 April 2015
Received in revised form 18 June 2015
Accepted 25 June 2015

Keywords:
Protein array
Allergens
Chemometrics
IgE
Horse

ABSTRACT

Insect bite hypersensitivity (IBH) is a seasonal recurrent skin allergy of horses caused by IgE-mediated reactions to allergens present in the saliva of biting insects of the genus *Culicoides*, and possibly also *Simulium* and *Stomoxys* species. In this work we show that protein microarrays containing complex extracts and pure proteins, including recombinant *Culicoides* allergens, can be used as a powerful technique for the diagnosis of IBH. Besides the obvious advantages such as general profiling and use of few microliters of samples, this microarray technique permits automation and allows the generation of mathematical models with the calculation of individual risk profiles that can support the clinical diagnosis of allergic diseases. After selection of variables on influence on the projection (VIP), the observed values of sensitivity and specificity were 1.0 and 0.967, respectively. This confirms the highly discriminatory power of this approach for IBH and made it possible to attain a robust predictive mathematical model for this disease. It also further demonstrates the specificity of the protein array method on identifying a particular IgE-mediated disease when the sensitising allergen group is known.

1. Introduction

Insect bite hypersensitivity (IBH) is a seasonal recurrent skin allergy of horses caused by IgE-mediated reactions to allergens present in the saliva of biting insects of the genus *Culicoides*, and possibly also *Simulium* and *Stomoxys* species (Fadok and Greiner, 1990; Wilson et al., 2001; Baselgia et al., 2006; Hellberg et al., 2006).

The estimated prevalence of IBH worldwide is variable, ranging from 3% to 11.6% in the UK (McCaig, 1973), 37.7% in parts of Germany (Littlewood, 1998), 10–60% in areas of Queensland, Australia (Riek, 1954) and 71.4% in some regions of The Netherlands (van Grevenhof et al., 2007). Interestingly, IBH is not found in Iceland, where *Culicoides* insects are absent, yet horses exported from Iceland for more than two years to countries where *Culicoides* are present show a prevalence of IBH of 50%, while the prevalence of IBH in their progeny born in mainland Europe is <10% (Björnsdóttir et al., 2006; Halldórdsóttir and Larsen, 1991).

Several IgE-binding salivary protein allergens from *Culicoides* have now been identified (for a review see Schaffartzik et al., 2012) in *C. nubeculosus* (Schaffartzik et al., 2010, 2011), *C. sonorensis* (Langner et al., 2009) and *C. obsoletus* (van der Meide et al., 2013; Peeters et al., 2013). Interestingly, despite the distinctively geographical distribution of the different *Culicoides* species, cross-reactivity has been observed amongst different species (Halldorsdottir et al., 1989; Anderson et al., 1993) but recent studies indicate that allergens from *Culicoides* species originating from the environment of the horses display higher IgE-binding than allergens from species only rarely present in this environment (van der Meide et al., 2012).

The clinical symptoms, together with a thorough medical history, taking other conditions that may lead to the characteristic pruritus of the disease into account, are still considered gold standard for the diagnosis of IBH. To confirm the clinical diagnosis,

* Corresponding author at: Division of Experimental Clinical Research, Department of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Bern, Länggass-str 124, CH-3001 Bern, Switzerland. Tel.: +41 31 631 23 30; fax: +41 31 631 26 30.
E-mail address: eliane.marti@vetsuisse.unibe.ch (E. Marti).

http://dx.doi.org/10.1016/j.vetimm.2015.06.013
0165-2427/

methods using crude whole body extract preparations as allergens have been used with mixed degrees of success such as intradermal skin test (Sloet van Oldruitenborgh-Oosterbaan et al., 2009; Wagner et al., 2009), *in vitro* histamine release (Wagner et al., 2008) and sulfidoleukotriene (sLT) release assays (Marti et al., 1999; Baselgia et al., 2006), and serological IgE tests (Frey et al., 2008; van der Meide et al., 2012). Recent studies indicate that the use of pure recombinant *Culicoides* proteins may result in an improvement of serological IgE tests for diagnosis of IBH (van der Meide et al., 2014; Peeters et al., 2013). However, because of the high number of *Culicoides* protein allergens relevant for IBH, component resolved diagnosis (CRD) using ELISA is cumbersome and expensive.

With the advances in robotics and computational technology, the ability to produce microarrays has provided the possibility to spot and monitor thousands of individual molecule populations on a miniaturised scale, on solid phase-based media of the size of a microscope slide. These protein spots and their interaction with specific molecules (e.g. immunoglobulins) can easily be tracked by techniques like fluorescence and advanced algorithms for image processing and pattern recognition. It is now well described and broadly accepted that beyond the comprehensive qualitative coverage, the diagnostic value of these tests with selected allergens can be similar to standard laboratory methods such as UniCAP, ELISA and immunoblot tests (Renault et al., 2011).

With the availability of a panel of recombinant *Culicoides* allergens, we demonstrate here that a complex protein array can be used for accurate diagnosis of IBH. Furthermore, we demonstrate that classification methods such as Partial Least Squares Discriminant Analysis (PLSDA), widely used by engineers and scientists in chemometrics-based research, can be a useful tool in the analysis of complex multivariate data allowing the production of predictive and testable mathematical models.

2. Materials and methods

2.1. Horses

A total of 133 horses comprising 63 non-affected (healthy) controls, 35 IBH-affected horses, 6 horses affected with recurrent airway obstruction (RAO) and 29 horses showing classic symptoms of recurrent urticaria were included in the study. The mean age of the horses was 11 years (range = 1–27). There were no significant differences between the groups except that the 6 RAO-affected horses were significantly older than the other horses (mean age = 19 years). The horses consisted of 56 females and 77 males, and again there were no significant differences in the gender distribution between the groups (Chi-Square = 0.6). The horses consisted of various breeds including Icelandic horses, Warmblood horses, Franches-Montagnes, Arabians, Andalusians and ponies.

In the first part of the study a subgroup of the above mentioned horses consisting of all 35 IBH-affected horses and of 37 healthy controls was analysed. In this group 24 out of the 35 IBH-affected horses and 25 out of the 37 non-affected horses belonged to the Icelandic breed. The IBH-affected horses had typical clinical signs of IBH and a clinical history of recurrent seasonal dermatitis affecting the dorsal and sometimes also the ventral midline. Furthermore, the IBH-horses were all positive in a cellular sLT release assay with *C. nubeculosus* extract, used to confirm the clinical diagnosis of IBH (Baselgia et al., 2006). The 37 healthy control horses had no clinical signs or history of skin diseases and were all living on the same farms where at least one of the IBH-affected horses was living, i.e. they were living in an environment where the causative insects for IBH (*Culicoides* spp.) were present. Furthermore, the healthy control horses had a sLT release with *C. nubeculosus* extract that was below the defined cut off (Baselgia et al., 2006).

In order to test the robustness of the test, in the second part of the study new microarray analyses were carried out. A larger group of randomly selected clinically non-affected horses and horses that were affected with conditions which are potentially IgE-mediated, including recurrent urticaria (Jose-Cunilleras et al., 2001; Rüfenacht et al., 2005; Hinden et al., 2012) and recurrent airway obstruction (Jose-Cunilleras et al., 2001) were analysed. The ability to discriminate IBH affected horses from other IgE-mediated disease background should demonstrate the robustness of the test.

2.2. IgE serum determination by protein microarray

A complex protein microarray containing extracts ($n = 240$) and pure proteins ($n = 120$) from a wide range of protein families from food (fruit, dairy, seeds), epithelium, pollen, fish, mollusca, fungi and insects representing in total 200 environmental and food species (Table S1) was assembled essentially as described previously (Wulfert et al., 2012; Renault et al., 2011). The extracts and purified proteins were obtained from commercial suppliers, produced in house and donated mainly by Dr. Maria Antonietta Ciardiello (Instituto of Biosciences and Bioresources, Napoli, Italy). Additionally for this study, a whole body *C. nubeculosus* extract available commercially (XPB681A2.5, Greer allergy Immunotherapy, Lenoir, USA) and a female *C. nubeculosus* thorax and head extract, made as described in Peeters et al. (2013), as well as recombinant *Culicoides* allergens were used. The recombinant *Culicoides* allergens originating from *C. nubeculosus* (Cul n 1–10) and *C. obsoletus* (Cul o 1 and Cul o 2) allergens were produced in *Escherichia coli* and purified as described previously (Schaffartzik et al., 2010, 2011; Peeters et al., 2013). IgE-binding to these *Culicoides* extracts and recombinant allergens were measured individually, but for the PLSDA analyses the same procedure used for all the other families of proteins contained in the array was employed, i.e. all *Culicoides*-specific data have been amalgamated and averaged. The purified protein solutions and extracts were normalised and spotted using a Marathon microarrayer (Arrayjet, Roslin, Scotland) into 16 pad nitrocellulose FAST slides (Whatman Schleicher & Schuell, Dassal, Germany) to a final density of 12,288 spots/slide. After blocking with 3% BSA (w/v) in PBS, the microarrays were hybridised with the horse sera diluted 1:2 in PBS containing 0.2% Tween-20 (PBST) and 2% BSA and surface bound allergen specific equine IgE was quantified using mouse anti-horse IgE mAb (Wilson et al., 2006) and fluorophore labelled rabbit anti-mouse IgG Ab (Dylight 649, Rockland Inc., USA) both diluted at 1:400 in 0.2% PBST containing 1% BSA. The slides were then washed three times in 0.05% PBST, followed by five times with purified water, and finally dried by centrifugation (MSE Mistral 3000i, Sanyo, UK) at $300 \times g$ for 10 min at room temperature as previously described (Renault et al., 2011). Dried slides were scanned in a Genepix 4000B (Molecular devices, USA) with the settings 440 and 310 for the PMTs at 635 and 532 nm, respectively, and image processed in GenePix Pro software v6.0.1.27 (Axon Instruments). Control microarray results, captured on individual slides (1 out of 16 pads), consisting of all reagents except horse serum, were subtracted from the sample slides to eliminate non-specific binding and inherent autofluorescence of some proteins using dedicated programs developed in house running on Matlab (Version 9, The Mathworks Inc., USA) using an Excel link toolbox (Mathworks) and Dataset Object (Version 5.0, Eigenvector Research Inc., USA).

2.3. Data analysis

Multivariate data analysis was carried out using the PLS Toolbox (Version 5.8.3, Eigenvector Research Inc., USA) with principal components analysis for data exploration/visualization and partial least square discriminant analysis software (PLSDA) for discriminant