

IgE ELISA using antisera derived from epsilon chain antigenic peptides detects allergen-specific IgE in allergic horses

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Abstract

Equine disease with an allergic etiology is common. Environmental antigens most often implicated as allergens in horses include molds, dusty hay, grass pollen, hay dust mites, and insect saliva. Although intradermal testing with allergen is a useful diagnostic tool for some species, skin testing frequently produces false positive results in horses. Allergen deprivation as a diagnostic tool is often impossible and at best it is ineffective at diagnosing the specific allergic reactivity. Synthesis of IgE after exposure to allergen is the instigator of the allergic process. While IgE exerts its effect after binding strongly to mast cell Fc receptors, the presence of free IgE in the serum can be used to quantify and determine the allergen specificity of the allergic disease. A lack of widely available reagents for detection of equine IgE has limited this approach in horses. We have used the nucleotide sequence of equine IgE to prepare a peptide-based immunogen to elicit equine epsilon chain-specific antisera. Selection of peptides was based on antigenic attributes of the deduced amino acid sequence of the equine epsilon chain. Six peptides were selected for conjugation to carrier molecules and rabbit immunization. Of these, one peptide elicited antisera that was successfully used in enzyme linked immunosorbent assay (ELISA) to screen horse serum from 64 allergic horses for allergen-specific IgE. Twenty-four of the 64 horses showed positive reactivity to one or more of the following allergens: grass, grain mill dust, mosquito, and horsefly. This study demonstrates the usefulness of peptide-based immunogens for development of antisera to rare or difficult to purify antigens such as IgE. Resultant antisera has great usefulness in diagnostic assays for equine allergy and as a research tool.

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1. Introduction

The importance of type 1 hypersensitivity in equine practice is considerable. Clinical diseases associated with IgE responses include anaphylactic shock

(following administration of routine biologicals such as vaccines), urticaria, allergic bronchitis, and chronic obstructive pulmonary disease (COPD). Despite the importance of allergy in equine practice, availability of reliable, standardized, serum-based testing for allergic reactivity has been poor to non-existent.

Due to the very low concentration of IgE in serum, purification of sufficient IgE for production of diagnostic reagents has been difficult. Nonetheless, [Halliwell and Hines \(1985\)](#) produced polyclonal anti-IgE using protein purification techniques ([Halliwell](#)

Abbreviations: COPD, chronic obstructive pulmonary disease; CH, constant region of heavy chain domain (CH1, CH2, CH3, etc.); P1–P6, peptide designation (P1, P2, P3, etc.); HS, horse serum

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and Hines, 1985). More recently, recombinant fusion proteins that mimic a piece of the intact protein have been used in lieu of purified serum IgE (Marti et al., 1997; Watson et al., 1997). However, both techniques require an extensive amount of labor and may not produce functional antisera. Synthesis of peptides based on continuous epitopes from the native protein is an alternative method for obtaining antigen for use in generating antisera. Indeed, functional antibodies against peptides have been shown to neutralize viral infectivity, indicating cross-reactivity to the native protein (Smyth et al., 1992). Based on the amino acid sequence of the epsilon chain, peptides can be generated by using algorithms to predict accessibility, hydrophilicity, and surface probability. Thus, peptides that are likely to be antigenic epitopes can be synthetically produced. Peptides serve as haptens and thus need to be conjugated to large carrier molecules to enhance their immunogenicity (Williams and Ibrahim, 1981). Production of antisera against peptides that show reactivity to native protein offers a more simplified method for the generation of antibodies against difficult to purify proteins.

Recently, the complete nucleotide and deduced amino acid sequence of IgE has been determined (Marti et al., 1995; Navarro et al., 1995; Watson et al., 1997). As a result, cloned segments of the epsilon chain genome have been used to create recombinant proteins produced in bacteria. Such proteins have been used to immunize several animal species. Resultant antisera have been shown to react with equine IgE under various conditions (Marti et al., 1997; Watson et al., 1997). However, conformational requirements such as natural folding of fusion proteins and perhaps a lack of glycosylation have made it difficult to generate polyclonal and monoclonal antibodies that recognize native equine IgE (Grioto-Wenk et al., 2000). Our group has created in *E. coli* a recombinant fusion protein consisting of parts of the third and fourth constant regions of the epsilon heavy chain (CH3 and CH4) fused with β -galactosidase. We have produced both polyclonal rabbit antibodies specific for equine IgE (Watson et al., 1997) and monoclonal antibodies (Pettigrew, unpublished data). However, the polyclonal antiserum was able to recognize only the reduced/denatured and non-reduced/denatured IgE but not the native IgE, particularly when bound to allergen in enzyme

linked immunosorbant assay (ELISA). Marti et al. (1997) also used a recombinant fusion protein to immunize chickens for production of antisera that reacted with equine IgE.

2. Materials and methods

2.1. Selection of peptides

In order to choose appropriate regions of the equine IgE heavy chain gene sequence to generate synthetic peptides, our deduced equine IgE heavy chain gene sequence was analyzed using the Garnier–Osjuthorp–Robson antigenic prediction, in the Genetics Computer Group, Inc. (GCG) model of Sequence Analysis Software Package (Jameson and Wolf, 1988; Devereux et al., 1984). The program's algorithm consists of subroutines analyzing amino acid sequence for hydrophilicity, surface probability, regional backbone flexibility, predicted secondary structure and flexibility parameters combined with hydropathic/solvent accessibility factors. This last subroutine was used to generate a linear surface contour profile (antigenic index) for the equine IgE heavy chain gene sequence (Fig. 1).

In deciding upon specific sequences of the heavy chain gene, consideration was placed predominantly upon regions of high antigenic index value along with high surface probability (Fig. 1). The gene region corresponding to the partially successful recombinant fusion protein product (a.a. 377–488) was avoided. Sequences were chosen such that each of the four constant regions was represented at least once. Sequence P1 (a.a. 332–346) is within the constant 2 (C2) region. Sequence P2 (a.a. 341–355) is within the C2 and C3 region. Sequence P3 (a.a. 153–167) is within the C1 region. Sequence P4 (a.a. 504–518) is within the C4 region. Sequence P5 (a.a. 286–300) is within the C2 region. Sequence P6 (a.a. 211–225) is within the C1 region. The chosen peptides and their designations are as follows: P1 (VTYKRHDLLRTRPRK) end portion of C2, P2 (RTRPRKCTESEPRGV) end portion of C2, P3 (LAACCKDTKTTNITL) beginning of C1, P4 (IQTDQQATTRPKSQW) early portion of C4, P5 (LIDGQKVDEQFPQHG) middle portion of C2, P6 (RVVASGKWAKQKFTC) latter portion of C1. All peptides were manufactured by Sigma Genosys, purified by HPLC, and provided lyophilized.

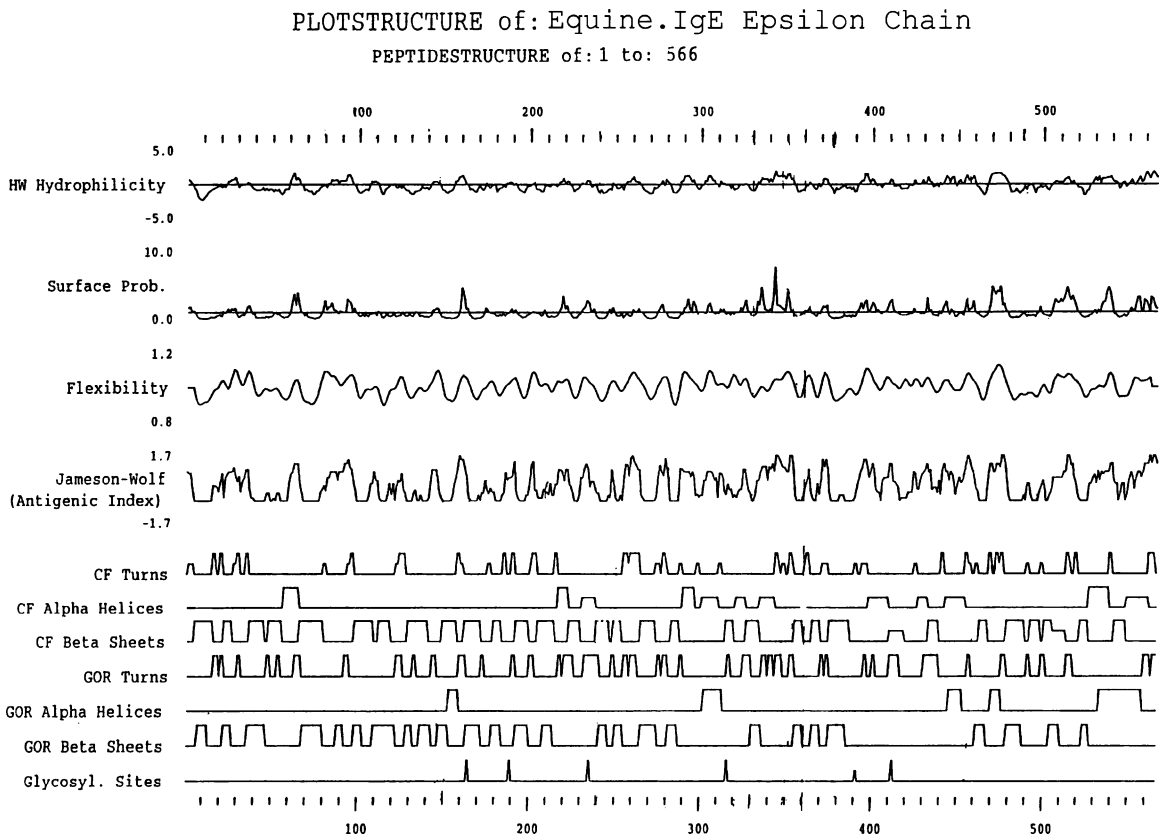


Fig. 1. Garnier–Osjuthorp–Robson antigenic prediction. The horizontal axis shows the primary deduced amino acid sequence of equine IgE. The vertical axis displays the parameters used to detect antigenic eptiopes.

2.2. Conjugation of peptides to keyhole limpet hemocyanin (KLH)

Each individual peptide was conjugated, via carboxyl groups, to KLH with the use of Imject Immunogen EDC Conjugation Kit with mcKLH (Pierce, 77602). Approximately 2 mg of peptide was conjugated to 2 mg of KLH by the carbodiimide EDC in 0.1 M MES, 0.9 M NaCl, pH 4.7 for 2 h. Removal of residual EDTA from the hapten–carrier mixture was accomplished by a gel filtration column of cross-linked dextran.

2.3. Immunization of rabbits with peptide–carrier conjugates

Eleven male and female New Zealand white rabbits were immunized separately with each peptide–carrier

conjugate. Six rabbits were immunized separately with 500 µg of P1–P6 in Freund's complete adjuvant (Gibco, 15721-030) and were boosted with 250 µg P1–P6 at 3-week intervals for a total of three boosts in Freund's incomplete adjuvant (Gibco, 15720-030). Three weeks after the third booster immunization, each rabbit was bled and serum was stored for future use.

2.4. Peptide-specific ELISA

An enzyme linked immunosorbant assay was performed to measure antibodies in antiserum that were specific to unconjugated peptides. Ninety-six well polystyrene plates were sensitized with 1 µg per well of each individual peptide in carbonate–bicarbonate buffer pH 9.6. Plates were then incubated overnight at 4 °C. On the following day, plates were blocked

with 1% rabbit serum albumin (Sigma, A-0639) in carbonate–bicarbonate buffer pH 9.6 and were incubated at 37 °C for 1 h. Plates were then soaked with a 0.1% Tween phosphate buffered saline (PBS) solution and washed vigorously five additional times. Antiserum to the respective peptide was added to each well at various dilutions in PBS and incubated for 1 h at 37 °C. Plates were washed as before. Secondary goat anti-rabbit IgG (H + L) horseradish peroxidase (HRP) conjugate (Zymed, 65-6120) diluted 1:10,000 was added and incubated for 1 h at 37 °C. Following a final wash, plates were developed with the use of *o*-phenylenediamine (Sigma, P1526) in 0.1 M sodium citrate pH 4.5. After 200 µl of substrate was added to each well, the plates were read on a Molecular Devices spectrophotometer (UVmax Microplate Reader) in dual wavelengths 450 and 650 nm.

2.5. Evaluation of peptide-specific antiserum using horse serum immunoblot

A 12% lower, 4% upper polyacrylimide gel was cast on a Mini-Protean Electrophoresis apparatus (Biorad). Horse serum from a horse with a clinical history of allergy was diluted 1:1 in reducing PAGE buffer. After completion, proteins were transferred to nitrocellulose with the use of a Mini-Protean Transblotter (Biorad). The nitrocellulose was then cut into strips and placed in 16 mm × 125 mm test tubes where they were subsequently blocked for 30 min with 3% skim milk (BBL, 11915). Rabbit anti-peptide antiserum was added at various dilutions to each tube and incubated overnight with the addition of 0.002% sodium azide at room temperature. Blots were then washed with a 0.2% Tween PBS solution three times. Secondary goat anti-rabbit IgG (H + L) HRP 1:1000 was incubated in the same manner. Blots were washed as previously described and were developed with 4-chloronaphthol substrate (Biorad, 170-6534).

2.6. Analysis of reactivity to equine IgE in two-dimensional (2D) immunoblot

The first dimension gel was composed of a 3% urea solution, 4% acrylamide solution with 3–10 pI amplo-cytes. Electrophoresis in tube gel format proceeded in a BioRad Tube Gel apparatus 175 and was run for 16 h at 700 V for first dimension separation of equine

serum proteins. The second dimension separation proceeded on a 10% SDS gel as described earlier. Immunoblot of gel proteins was carried out as described earlier. As a control, mouse myeloma IgE (BD-Pharmingen) was run concurrently during each step and stained in the same manner for reproducibility of mobility for IgE. Immunoblots were stained with either anti-P5 antibody, or goat anti equine IgG (Bethyl Labs.), or goat anti-mouse IgE (ICN Biochemicals).

2.7. Reactivity of peptide-specific antiserum to ovalbumin-specific equine IgE in ELISA

An antigen-specific ELISA was performed similar to the peptide-specific ELISA. Microtiter wells were sensitized with 1 µg of ovalbumin (Sigma, A-5503) and blocked with 0.5% rabbit serum albumin. After an hour, incubation plates were washed as previously described. Serum from ovalbumin immunized horses (kindly provided by Dr. David Horohov, School of Veterinary Medicine, Louisiana State University) was added to each ovalbumin sensitized well at a dilution of 1:4. Pre-ovalbumin immunization sera were used as negative controls and diluted the same. Secondary anti-peptide antisera was used at various dilutions and incubated as described above. Following a brief wash, goat anti-rabbit HRP was added and incubated. After a vigorous wash, plates were developed and read as previously described.

2.8. Purification of peptide-specific antibodies by affinity chromatography

Affinity purification of peptide-specific antisera was accomplished using EDC/Diaminodipropylamine Immobilization Kit (Pierce, 44899). Five milligrams of peptide was conjugated to diaminodipropylamine with the use of EDC according to the manufacturer's instructions. Following conjugation each peptide column was equilibrated with PBS pH 7.4. The immunoglobulins were purified from 5 ml of hyper-immunized rabbit serum by precipitation with 50% saturated ammonium sulfate, concentrated to 1 ml, and dialyzed against PBS. Next, 1 ml of serum was added to the column and allowed to incubate for 1 h. Following incubation, the column was washed with 15 ml of PBS and then eluted with 0.1 mM

glycine pH 3.0. Low pH in tubes with eluted protein was neutralized with one part of 10× phosphate buffered saline solution.

2.9. Standardized allergen-specific equine IgE ELISA

Serum from 76 horses with various clinical signs of allergy was obtained from cooperating veterinarians. These samples were tested in an antigen/allergen-specific IgE ELISA against the following seven antigens/allergens: mold mix (Greere M03-34-1F4), GS7 grass mix, (P27-40-3F14), western tree mix (P39-1-1F2), grain mill dust mix (D02-22-1X2), mosquito mix (B55-8-1P1), mite mix (B060-73-1C1), and horsefly mix (B43-21-1F4). Each ELISA was performed the same as the ovalbumin-specific ELISA, except the rabbit anti-peptide antibody P5 affinity purified was the chosen anti-IgE reagent and serum from each horse was added undiluted. For each antigen a pool of horse serum from eight non-allergic horses was used for a negative reference control. Values on the ELISA were recorded as percent of negative control where anything above 200 was considered positive.

3. Results

3.1. Antibodies derived against peptides in ELISA

Each respective peptide immunized rabbit produced antisera against the corresponding peptide. Results of the peptide-specific ELISA are presented in Table 1. Titers to individual peptides varied but all rabbits made a response to their respective peptide and carrier (data not shown).

3.2. Peptide derived antibodies in equine IgE immunoblot

Immunoblots were performed on IgE purified from allergic horse serum by 50% saturated ammonium sulfate fractionation followed by size exclusion dialysis using cellulose ester dialysis tubing at 100,000 Da MW cut-off. Fig. 2 demonstrates reactivity of peptide-specific antisera with denatured reduced equine IgE on horse serum immunoblot. All peptides

Table 1
Peptide-specific ELISA performed on rabbit serum

Peptide no.	OD pre-immune ^a	OD post-immune ^a	P/N ratio ^b
1	0.016	0.211	13.2
2	0.023	0.109	4.46
3	0.019	0.179	9.42
4	0.015	0.070	4.67
5	0.018	0.275	15.3

^a Average optical density read in dual wavelength at 450 and 650 nm.

^b P/N is a ratio of post-immunization OD to pre-immunization OD where P/N = 2.0 is considered positive.

were derived against continuous epitopes; therefore, it is not surprising that peptide-specific antisera recognized continuous, whole heavy chain IgE at 65 kDa. P1, P2 and P3 antisera consistently produced the densest bands in immunoblot even though this does not correlate with the titers of peptide-specific antisera in ELISA. P5-specific antisera reacted to a single 65 kDa band but with lesser intensity than P1, P2, and P3. P4 and P6 antisera reacted with a band that correlated to the molecular weight of IgE but they reacted with other bands as well. This was consistent even after affinity purification. Secondary antibodies (conjugates) did not react to equine proteins.

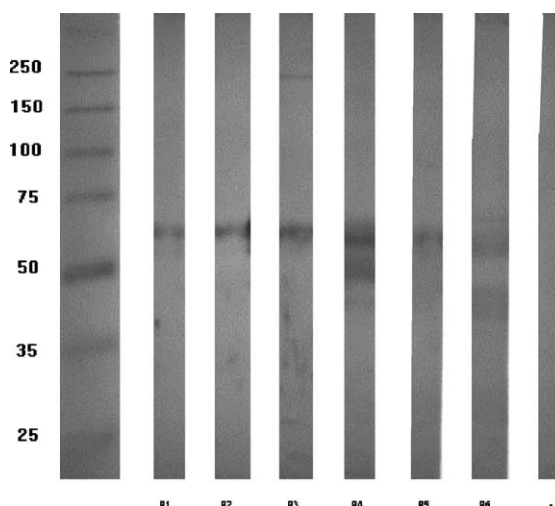


Fig. 2. Immunoblots of reduced/denatured equine IgE and reactivity of individual peptide antisera. A solid band at ~65 kDa is seen in all blots.

3.3. Reactivity of anti-P5 in equine IgE 2D immunoblot

Fig. 3 shows the results of 2D separation of equine serum proteins. The results from the 2D blots were intended to prove that anti-P5 did not recognize equine IgG, which has a different isoelectric point and molecular weight than IgE. The conditions of the second dimension gel were also non-reducing. This contributed to better separation of equine IgG from IgE, since the difference in molecular weight is 38 kDa. Fig. 3 shows a blot recognized by anti-P5 which is considerably heavier than equine IgG; furthermore, the position on the blot corresponds to the position of mouse IgE when separated under the same conditions. The isoelectric shift, noted by separation in the first dimension (left to right on the gel), was different for the P5 reactive blot (IgE) than that for the blot of IgG (Fig. 3). The shift in the anti-P5 recognized blot is also

comparable to the shift seen in the myelomic mouse IgE 2D immunoblot. Therefore, in this experiment identification of mouse myeloma IgE in a 2D immunoblot, by a polyclonal anti-mouse IgE, was used as a positive reference control.

3.4. Equine serum immunoblot comparison of anti-P5 with anti-fusion protein

Both rabbit anti-P5 and rabbit anti-fusion protein (recombinant CH3 and CH4 heavy chain equine IgE) recognize a band of similar molecular weight (65 kDa) on a reduced denatured equine serum immunoblot (Fig. 7).

3.5. Ovalbumin-specific equine IgE ELISA

To test for reactivity to IgE once it has bound to antigen, ovalbumin-specific serum was tested in

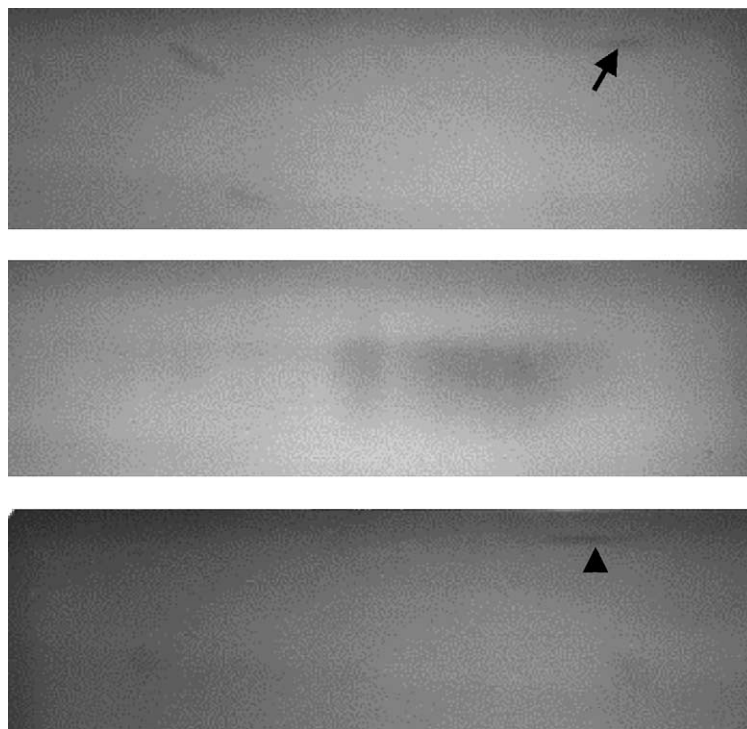


Fig. 3. 2D equine serum immunoblot. The top figure illustrates anti-P5 reactivity to IgE in a second dimension gel (arrow). The middle figure illustrates the position of equine IgG, detected by a goat anti-equine IgG heavy chain only. The bottom figure shows the position of murine IgE in an immunoblot containing only mouse myelomic IgE and stained with an anti-mouse IgE (arrowhead). The murine IgE serves as a reference for isoelectric position and molecular weight of epsilon chain for comparison with our anti-P5's reactivity to equine IgE.

ELISA. Serum from ponies before ovalbumin immunization and aerosolization were used as a negative reference control and pre-peptide immune rabbit serum was also used as a negative control. Table 2 shows the data expressed in optical density and positive to negative ratio. P1 and P5 recognized antibody bound to ovalbumin; however, P2, P3, P4 and P6 failed to recognize native IgE in this ELISA. In another ELISA (data not shown), P1 antisera reacted strongly, in ELISA, to purified SAS cut horse immunoglobulins that were diluted beyond the point of probable IgE detection; whereas, anti-P5 did not react.

Table 2

Ovalbumin-specific IgE ELISA performed on equine serum

Peptide no.	OD pre-OVA ^a	OD post-OVA ^a	P/N ratio ^b
1	0.029	0.130	4.48
2	0.017	0.016	0.94
3	0.015	0.024	1.60
4	0.024	0.026	1.08
5	0.026	0.079	3.04
6	0.026	0.024	0.92

^a Average optical density read in dual wavelength at 450 and 650 nm.

^b P/N is a ratio of post-OVA sensitization value/pre-OVA sensitization value where P/N = 2.00 is considered positive.

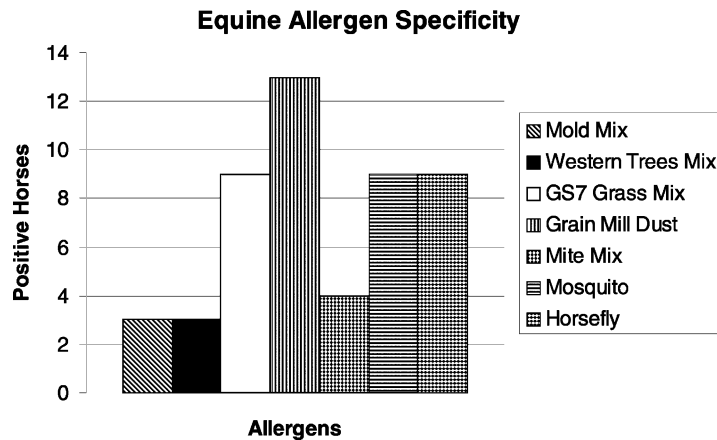


Fig. 4. The bar graph illustrates the number of horses that had S/N ratios of 2 or higher on IgE peptide-based ELISA using seven allergens.

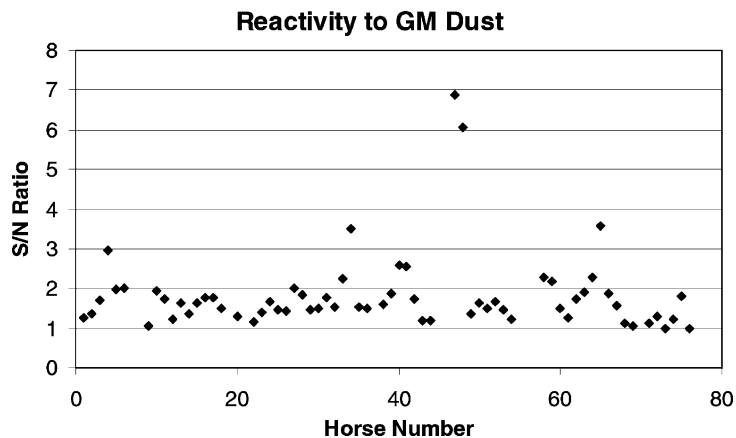


Fig. 5. Sixty-four horses were screened against grain mill dust in peptide-based ELISA. The horizontal axis shows specimen number and the vertical axis shows the level of reactivity with respect to non-allergic (normal) horses (S/N ratio). Sample numbers included 76 horses; however, data was not obtained from 12 horses due to insufficient quantity or poor quality of the sample submitted.

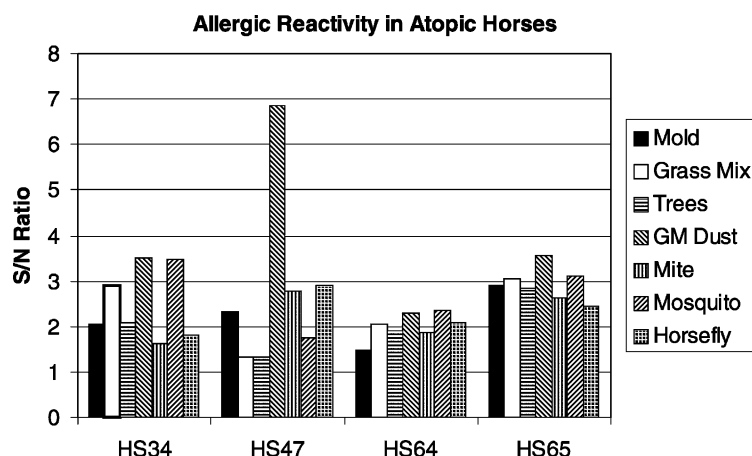


Fig. 6. Display of allergen-specific IgE reactivity in atopic horses. The four horses displayed here show positive reactivity for most of the allergen mixes and all exhibited signs of urticaria.

3.6. Allergen-specific equine IgE ELISA

Seventy-six horses with clinical signs of allergy were bled by veterinarians throughout the Western and Mid-western US. These serum samples were tested in an allergen-specific IgE ELISA using affinity purified anti-peptide antibodies. Several common mixes of allergens were used to deduce probable allergen sensitivities. Among the common mixes were molds, western grasses, western trees, grain mill dust mix, mite mix, mosquito, and horsefly. The results from 64 of the 76 horses are summarized in Fig. 4. Assessment of horses that were positive for any given allergen was based on a comparison of horse serum from a pool of eight normal horses. Sample to negative (S/N) ratios were used to determine a reactivity score in the following way: negative reactivity <1.5, borderline reactivity between 1.50 and 1.99 positive reactivity between 2.00 and 4.00, and high reactivity >4.00. Fourteen horses showed positive reactivity to grain mill dust, nine displayed reactivity to grasses, horsefly, and mosquito, four to mites, and three were positive against molds or western trees. Fig. 5 shows the frequency of positive reactivity to grain mill dust (most common allergen) on a per horse basis. Using S/N ratios to score reactivity, 25 horses were borderline, 12 positive, and 2 high positive for grain mill dust. Severely atopic horses were defined as showing reactivity to at least four out of the seven allergens. Four horses displayed positive reactivity to multiple

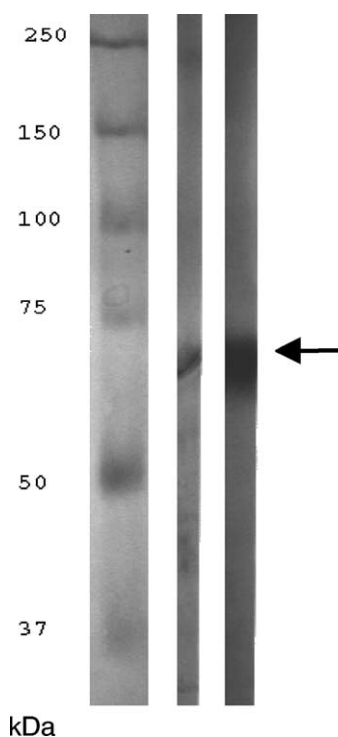


Fig. 7. Equine IgE Immunoblot with anti-P5 and anti-fusion protein (CH3 and CH4 heavy chain equine IgE). The immunoblot (right) illustrates reactivity of a previously published polyclonal antibody generated against a recombinant protein expressing the CH3 and CH4 portion of equine IgE. Both the anti-fusion protein polyclonal and affinity purified anti-P5 polyclonal (left) react to a protein in equine serum of similar molecular weight (arrow).

allergens; the results are summarized in Fig. 6. Fig. 6 also shows that all atopic horses had antibodies reactive with grain mill dust.

4. Discussion

Equine allergy is a constant problem for veterinarians and horse owners (Fadok, 1995; Swiderski, 2000). Although reagents detecting equine IgE have been developed, their use in diagnosis has not been widely exploited. In this study, we successfully produced native equine IgE reactive antiserum from peptide immunized rabbits. Continuous epitopes can either be cryptic or exposed; however, the use of multiple algorithms that predict surface exposure, allowed us to choose peptide sequences that were likely to be assessable. Although all of the chosen peptides produced functional antiserum that reacted with reduced denatured IgE, only two recognized IgE in its native form in ELISA. Furthermore, of these two peptides, antiserum against P1 showed excessive background in ELISA when total equine immunoglobulins were sensitized on the plate beyond the point of probable IgE detection. This could indicate that the P1 epitope is cross-reactive with discontinuous epitopes of other equine immunoglobulins. This would also explain why P1 antisera appears to work much better in the ova-specific ELISA especially since other antibody isotypes were likely also bound to the ovalbumin. (Table 2). In contrast, P5 antisera did not show any reactivity to other equine immunoglobulins in the same ELISA format. Incidentally, immunoblots of IgE revealed a light band at 65 kDa for P5 antiserum, but several bands were apparent for P4 and P6 antisera even after affinity purification. The excessive amount of reactivity could either indicate cross-reactive epitopes on denatured/reduced serum proteins or contaminants present in the synthetic peptide preparation, that mimic cryptic epitopes of serum proteins, that were used to immunize the rabbits. However, it is clearly apparent, by 2D immunoblot, that P5 antiserum does not show any reactivity to equine IgG. The position of the reactive spot on the 2D immunoblot is also consistent with the position, in terms of isoelectric point and molecular weight, of murine IgE (Fig. 3). P5 antisera also reacted, in an equine immunoblot, to the same 65 kDa band that our previously published anti-

equine IgE (anti-fusion protein antibody) reacted to (Figs. 6 and 7) (Watson et al., 1997). Therefore, our selection of P5 antisera in the preceding experiments was based primarily on its recognition of IgE in immunoblot and ELISA and on its lack of signal production in an IgE absent ELISA that contained IgG.

In summary, we have demonstrated that the antiserum prepared against a peptide (P5) chosen from the deduced amino acid sequence of the equine epsilon immunoglobulin heavy chain specifically reacts with equine epsilon chain, and hence IgE. Evidence for this comes from: (1) recognition of a 65 kDa band on an immunoblot from allergic horse serum corresponding to the band elicited by rabbit anti-fusion protein (previously published anti-IgE reagent), (2) recognition of epsilon chain spot on 2D immunoblot (non-reduced, denatured) of allergic horse serum at the same site on the gel as murine IgE myeloma-derived epsilon chain, together with non-recognition of equine gamma chain on the same gel, (3) lack of binding to other Ig classes on IgE-absent ELISA, and (4) detection of ovalbumin-specific IgE in sera from ovalbumin-allergic ponies. Moreover, this antiserum effectively defined specific allergen reactivity in clinically allergic horses when used in ELISA. The antiserum was not tested in ELISA for the ability to differentiate heat-inactivated serum from non-heat-inactivated serum because the P5 epitope is present on the middle portion of C2. This portion of IgE is not associated with heat denaturation of IgE receptor binding activity (Dorrington and Bernich, 1978).

The use of synthetic peptides that mimic continuous epitopes on native proteins offers an alternative to conventional techniques of generating antibodies to proteins that are difficult to purify, such as IgE. Furthermore, although the monospecific nature of anti-peptide antibodies may decrease sensitivity, non-specific reactivity and high backgrounds are also diminished when affinity purified antibodies are used (Fig. 7). Our data show that affinity purification of the anti-peptide antibody is necessary when screening sera against antigens that are ubiquitous, such as many allergens. This procedure eliminates the possibility that rabbit antiserum may react not only to the P5 epitope, but also directly to antigen in ELISA, thereby causing false positive results.

All results from the equine IgE ELISAs were essentially based on a comparison ratio of allergy negative horses. These horses had no clinical signs

of allergy and no reported history of allergy and were, therefore, considered negative. All high responding horses in our panel displayed urticaria, which was usually seasonal. Several horses showed only borderline or negative reactivity of IgE to allergens. Most horses diagnosed with chronic obstructive pulmonary disease did not show any antigen-specific IgE in sera. Other researchers have seen similar results in which antigen-specific IgE was not found in serum but was present in bronchoalveolar lavage fluid (BALF) (Halliwell et al., 1993; Schmallenbach et al., 1998). In general, the timing for sampling of serum for analysis of circulating IgE is critical, since the IgE half-life is short (Hirano et al., 1983). Thus, this difference in detection of IgE in urticaria versus COPD in horses may reflect the acuteness of urticaria episodes versus the chronicity of COPD.

The usefulness of the peptide-based IgE ELISA is further demonstrated by the following observations based on serum IgE testing. Fig. 4 shows that a mixture of grain mill dust was a common antigen for which IgE was produced and Fig. 5 shows that most horses scored as either borderline positive or positive for this allergen. The grain mill dust consisted of a mixture of barley, oats, corn, and wheat; all of which are commonly exposed to horses on a daily basis. Insect hypersensitivity was also commonly seen in the panel of horses, with mosquito and horsefly sensitivity most common. Mite sensitivity was less common and samples that were positive for mite were usually also positive for mosquito, suggesting cross-reactivity. The most common clinical signs reported for mosquito or horsefly IgE positive horses were urticaria, pruritis, and raised skin nodules—all consistent with insect hypersensitivity.

Grass allergy was also common among the horses tested. Clinical descriptions of horses that showed IgE positive reactivity to grass mix, generally, had more generalized urticaria than insect IgE positive horses. However, relating clinical signs with serum-specific IgE is difficult because some of the horses that were positive for grass were also positive for certain insects. This is consistent with the observation shown in Fig. 6 in which the horses tested displayed positive IgE levels to several different allergens. These horses could be classified as atopic. Thus, 4 of the 64 horses appeared to be atopic in that they produced positive results for IgE against 4 of the 7 allergens. One of

these four horses was positive against all seven allergen mixes and was reported to have extensive urticaria. Whether or not these atopic horses manifest clinical allergy to all allergens to which they are positive by ELISA or only to a select few (those to which they are highly positive) remains to be elucidated.

Allergen-specific serum IgE levels are a good indicator of allergy and have been used to assess allergic disease in humans and in dogs (Mueller et al., 1999; Halliwell, 1973). However, the lack of proper tools for its detection has precluded its use in equine medicine. In the present study the peptide-based anti-IgE reagent recognized allergen-bound IgE and generated descriptive statistics from horse sera analyzed by ELISA. Thus, from a sample base of 64 complete submissions (history and serum) 35% of the horses reacted positively to at least one allergen. Many of the horses that did not react with a positive score on any allergen showed borderline reactivity to one or more allergens. For some this may represent a waning IgE serum titer, since seasonality of allergic disease may have caused diminution of serum IgE levels. Of the reactive horses 65% had clinical signs of urticaria; some also had seasonal pruritis (midline dermatitis). In contrast only 18% of samples submitted were from horses with clinical signs of respiratory allergy (cough with or without COPD). The allergen that was most frequently implicated in disease expression was grain mill dust. Seventy-three percent of the horses that reacted to grain mill dust had signs of urticaria, and both of the horses with respiratory disease that tested positive to any allergen reacted to grain mill dust. Using the peptide-based anti-IgE ELISA we were able to discriminate four levels of reactivity: negative, borderline, positive, and strongly positive, allowing us to discriminate specific reactivity in clinically allergic horses.

We propose that the peptide-based methodology for production of anti-IgE is a useful technique that could be applied to development of anti-IgE reagents in various other species.

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