



Research paper

Intra and inter-laboratory reproducibility of a monoclonal antibody cocktail based ELISA for detection of allergen specific IgE in dogs: Proficiency monitoring of *macELISA* in six laboratories

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ARTICLE INFO

Article history:

Received 9 August 2011

Received in revised form 7 May 2012

Accepted 9 May 2012

Keywords:

ELISA

Allergen-specific IgE

Atopy

Dog

ABSTRACT

The purpose of this study was to evaluate the reproducibility of results yielded using a monoclonal antibody based ELISA for detection of allergen specific IgE when run in six separate affiliated laboratories. On two separate occasions, duplicate samples of 15 different sera pools were independently evaluated by each laboratory in a single blinded fashion. The average intra-assay variance among reactive assay calibrators in all laboratories was 6.2% (range 2.6–18.2%), while the average intra-laboratory inter-assay variance was 12.1% (range 8.0–17.1%). The overall inter-assay inter-laboratory variance was consistent among laboratories and averaged 15.6% (range 15.1–16.6%). All laboratories yielded similar profiles and magnitudes of responses for replicate unknown samples; dose–response profiles observed in each of the laboratories were indistinguishable. Considering positive/negative results, inter-assay inter-laboratory concordance of results exceeded 95%. Correlation of OD values between and among all laboratories was strong ($r > 0.9$, $p < 0.001$). Correlation of OD values between the two separate evaluations was also high for all allergens except olive, which was attributed to lot-to-lot differences of allergen coated wells. Collectively, the results demonstrated that the monoclonal antibody based ELISA for measuring allergen specific canine IgE is reproducible, and documents that consistency of results can be achieved not only in an individual laboratory, but between laboratories using the same monoclonal-based ELISA.

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1. Introduction

Allergy in dogs often presents as a chronic pruritic skin condition that requires long-term management to

alleviate and prevent the recurrence of clinical signs (Olivry and Sousa, 2001; Reedy et al., 1999a,b; Griffin and DeBoer, 2001; Griffin and Hillier, 2001; Gorman and Halliwell, 1989). Allergen-specific immunotherapy, with or without concurrent use of anti-inflammatory drugs, is often the method selected for intervention in this disease (Olivry and Sousa, 2001; Griffin and Hillier, 2001). The selection of allergens included in the allergen specific

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therapeutic regimen relies on demonstration of IgE-mediated hypersensitivity by either intradermal skin testing (IDT) or allergen-specific IgE serology (Olivry and Sousa, 2001; DeBoer and Hillier, 2001a,b; Hill DeBoer and Hillier, 2001). Unfortunately, there is a paucity of useful information that defines the comparative utility of the various allergen-specific IgE assays within the veterinary arena, and no definitive information exists that demonstrates consistency of results among the various veterinarians who perform IDT (Halliwell and DeBoer, 2001; Hill and Olivry, 2001; Bond et al., 1994; Codner and Lessard, 1993; Hammerling and De Weck, 1998). In evaluating serum based testing, the American College of Veterinary Dermatology Task Force on Canine Atopic Dermatitis (currently International Task force on Canine Atopic Dermatitis (ITF-CAD) reported that methodology for these tests varies by laboratories; few critical studies have evaluated the performance of these tests, and current inter-laboratory standardization and quality control measures are inadequate (DeBoer and Hillier, 2001b).

Since then, we have described the performance characteristics of a monoclonal antibody cocktail-based ELISA (*macELISA*) and provided a preliminary inter-laboratory evaluation between two laboratories that run this assay (Lee et al., 2009). Not only have we documented inter-laboratory consistency of results between laboratories that perform *macELISA*, we have shown that similar results are observed between our assay and a laboratory (Heska Corporation, Fort Collins, CO) that performs an assay using the alpha chain of human high affinity IgE receptor (Fc ϵ R1; *fceELISA*) (Stedman et al., 2001). More recently, an independent peer-reviewed study demonstrated acceptable intra- and inter-laboratory variability among three laboratories that use *fceELISA* (Thom et al., 2010). Consistent with previous studies (Lee et al., 2009), these investigators confirmed that variability is most prominent and of clinical relevance with low values that are around the cut-off point.

The present study was designed to provide a longitudinal comparative evaluation of the results observed with *macELISA* in five European Laboratories with those of Greer's reference laboratory located in Lenoir, NC by monitoring not only the concordance of results but the intra- and inter-assay variances evident within each laboratory as well as the inter-laboratory variances evident among the laboratories.

2. Materials and methods

All serum samples, buffers, coated wells, calibrator solutions, and other assay components were aliquants of the respective lots of materials manufactured at Greer's production facilities (located in Lenoir, NC, USA) and supplied as complete kits to the participating laboratories along with the exact instructions for completing the evaluations. Separate proficiency evaluations were completed in June, 2009 and March, 2010.

2.1. Participating laboratories

Five independent Veterinary Reference Laboratories (VRLs) participated in the initial proficiency evaluation of

macELISA; an additional European Laboratory joined this group during the second proficiency evaluation. Participating European laboratories included Agrolabo (Scarmagno, Italy), Artu Biologicals (Lelystad, The Netherlands), Axiom Laboratories (Newton-Abbott, UK), Univet Diagnostic Services (Barcelona, Spain), and Biovac (Beaucouzé, France). The VRL at Greer Laboratories (Lenoir, NC) served as the prototype for evaluation of *macELISA*. Because the performance characteristics of *macELISA* in Greer's VRL have been well documented for use over an extended period (Lee et al., 2009), all results observed in the other participating laboratories were compared directly with the results observed in Greer's reference laboratory.

2.2. Serum samples

Eight separate pollen and mite reactive serum pools and two non-reactive sera pools were prepared from samples that previously had been evaluated using *macELISA*. The allergen specific reactivity of each sera pool ranged from nonreactive to multiple pollen or mite reactivity. These sera pools and admixtures of the pools were used to construct a specific group of samples that exhibited varying reactivity to the allergens included in the evaluation panel. In addition, a three-fold serial dilution of a highly reactive pool, diluted into non-reactive sera, served to document the dose-response evident within the assay. Fifteen samples were included in each blinded evaluation and the samples for both were identical; but the samples were uniquely identified for the separate evaluations. Two pollen reactive control samples and one non-reactive control sample were also included in the second evaluation.

2.3. Calibrators

Calibrator solutions of predetermined reactivity in the *macELISA* were prepared as two-fold serial dilutions (calibrators #1–4) of a sera pool highly reactive to most pollen allergens. An additional calibrator (#5) was prepared from sera shown to be negative to pollen allergens in the *macELISA*. The reactivity of these calibrators to a mixture of grass pollen allergens was determined in each assay run. The expected uncorrected for background values for each of the calibrators 1–5 was 2346, 1246, 942, 534, and 122 EAU, respectively.

2.4. Buffers

The buffers used throughout have been previously described (Lee et al., 2009), and included: (a) well coating buffer: 0.05 M sodium carbonate bicarbonate buffer, pH 9.6; (b) wash buffer: phosphate buffered saline (PBS), pH 7.4, containing 0.05% Tween 20, and 0.05% sodium azide; (c) serum and reagent diluent buffer: PBS, pH 7.4, containing 1% fish gelatin, 0.05% Tween 20 and 0.05% sodium azide.

2.5. Allergen panel

The allergen panel used for proficiency evaluations in each of the laboratories was a 24 allergen composite derived from the array of allergens that are included in all of

the panels used in the European laboratories. The composite allergen panel consisted of 4 grasses, 6 weeds, 6 trees, 5 mites, and 3 fungi.

2.6. Preparation of coated wells

Immulon 4HBH flat bottom strip assemblies (Thermo Electron Corporation, Waltham, MA, USA) were used throughout and served as the solid phase for all direct bind ELISA. The 12-well strips were individually coated with the specified allergen extracts following a previously defined procedure (Lee et al., 2009). Briefly, the individual extracts were diluted in bicarbonate buffer (pH 9.6) and 100 μ L was added to each assigned well. Following overnight incubation at 4–8 °C, the wells were washed with PBS, blocked with 1% monoethanolamine (pH 7.5) then air dried and stored at 4–8 °C in Ziploc bags until used.

2.7. Sample evaluations – macELISA

The specific IgE reactivity to the various allergens included in the proficiency panel evident in each of the serum pools was determined using the direct bind macELISA (Lee et al., 2009). Briefly, 100 μ L of appropriately diluted sample (1:6) was added to duplicate wells that had previously been coated with the various specific panel defined allergens. Following an overnight incubation (16–18 h) at 4–8 °C in a humidified chamber, the wells were washed (2 complete aspirate/wash cycles using PBS

wash solution), then 100 μ L of an appropriately diluted cocktail of biotinylated monoclonal anti-dog IgE second antibody was added to each well. The wells were returned to the humidified chamber and incubation continued at room temperature (20–25 °C) for another 2 h, and then they were washed (3 complete aspirate/wash cycles). Streptavidin–alkaline phosphatase enzyme conjugate was added and incubation at room temperature continued for 1 h. Following a final wash step (4 complete aspirate/wash cycles) 100 μ L of *p*-nitrophenylphosphate substrate (*p*NPP) was added to each well and incubation continued for precisely 1 h. Substrate development was then stopped by adding 50 μ L of 20 mM cysteine to each well. Specific IgE reactivity to the allergens was estimated by determining the absorbance of each well measured at 405 nM using an automated plate reader. All results were expressed as ELISA Absorbance Units (EAU) which is background corrected observed responses expressed as milli absorbance.

2.8. Statistics

A coefficient of variation was calculated as the ratio of standard deviation and means of the responses observed for the calibrator solutions within different runs in multiple laboratories. Deming correlation analysis (MedCalc Software 12.2.1.0, Broekstraat 52, 9030 Mariakerke, Belgium) was used for overall inter-laboratory comparison. Pearson's correlation statistic was used for inter-laboratory comparison among individual allergens.

Table 1

Assay variance of macELISA calibrator solutions observed with different runs in multiple laboratories.

Variance		N	Calibrator ^b % CV					Background ^a % CV
			No. 1	No. 2	No. 3	No. 4	No. 5	
Inter-laboratory		378	16.6	15.3	15.3	15.1	25.0	46.9
Inter-assay								
Greer		156	11.6	10.6	9.5	9.1	11.2	17.8
ARTU		66	14.2	10.7	12.3	11.6	20.3	24.0
Axiom		44	14.7	17.1	15.9	15.3	32.7	56.7
Agrolabo		44	13.2	13.0	13.6	12.0	8.9	11.7
Univet		44	8.0	11.4	9.0	9.6	11.7	22.0
Intra-assay								
Greer	Trial #1	24	9.9	8.8	10.7	6.3	20.3	24.8
	Trial #2	24	5.6	7.3	7.9	8.6	6.2	7.1
	Trial #3	20	4.2	5.6	4.3	5.0	8.0	13.9
	Trial #4	24	6.1	7.1	4.8	5.3	6.5	22.8
	Trial #5	20	4.2	4.1	4.2	4.5	6.7	12.2
	Trial #6	24	6.2	6.7	6.3	6.9	4.9	10.2
	Trial #7	20	4.7	4.2	5.3	3.8	9.5	12.4
ARTU	Trial #1	18	4.8	8.6	6.4	11.0	27.5	29.1
	Trial #2	24	6.4	5.3	5.0	5.4	15.6	18.5
	Trial #3	24	8.4	8.9	7.7	8.4	16.1	23.6
Axiom	Trial #1	20	6.0	6.5	6.0	18.2	24.0	34.8
	Trial #2	24	7.3	5.7	6.3	6.3	27.2	13.3
Agrolabo	Trial #1	20	8.4	6.3	8.4	7.0	5.7	7.6
	Trial #2	24	6.4	9.2	7.4	8.4	10.9	13.8
Univet	Trial #1	20	2.6	5.9	6.8	5.2	14.4	13.7
	Trial #2	24	6.4	10.6	8.4	7.3	8.7	27.1
BioVac	Trial #1	24	7.4	8.3	10.0	8.5	5.8	8.3

^a Responses observed with diluent in place of serum sample.

^b The reference calibrators were prepared from a stabilized canine serum pool that contains a high level of IgE-specific for grass pollens. Calibrators 1–4 are prepared as a two-fold serial dilution of the serum pool, and calibrator 5 is prepared from a known negative serum pool.

3. Results

The assay variance (% CV) observed with the calibrator solutions in the different laboratories are presented in Table 1 and are representative of the assay reproducibility in the various laboratories. The average intra-assay % CV among positive calibrators (#1–4) was 8.0% (range 2.6–18.2%); substantial differences among laboratories or between assay runs were not detected. Intra-assay variability was higher with negative calibrator #5 (average 12.8%; range 4.9–27.5%), while the greatest intra-assay variability was evident with the background ODs (average 17.2; range 7.1–34.8%). The average inter-assay variance (% CV) observed with the calibrator solutions within each laboratory among the positive calibrators (#1–4) was 12.1% (range 8.0–17.1%). Inter-assay variability was higher with negative calibrator #5 (average 17.0%; range 8.9–32.7%), while the greatest inter-assay variability was evident with the background ODs (average 26.4; range 11.7–56.7%). The inter-laboratory % CV among reactive calibrators (#1–4) remained relatively constant (average 15.6%; range 15.1–16.6%). Again, the greatest variance among the various laboratories was observed with the negative calibrator #5 (25.0%), and with the background ODs (average 46.9%).

The concordance of positive and negative results observed within and between laboratories is presented in Table 2. Using predefined cut-off points of 150 EAU, 300 EAU, and 600 EAU results were classified as

positive or negative. The average intra-assay concordance of results across both proficiency evaluations using the 150 EAU cut-offs was 94.9% (range 89.2–97.2%). Similarly, the average inter-assay intra-laboratory concordance of results was 95.1% (range 92.2–96.8%), and the inter-assay inter-laboratory concordance of results was 95%. Increasing the positive–negative cut-off level to 300 EAU or 600 EAU appreciably increased the concordance of observed results. The average Intra-assay concordance of results at a cut-off of 300 EAU was 97.7% (range 93.3–99.3%) while the observed concordance at a cut-off of 600 EAU was 98.8% (range 97.6–99.8%). The average inter-assay intra-laboratory concordance of results was demonstrated to be 97.9% (range 95.5–99.3%) at a cut-off of 300 EAU and 98.8% (range 98.0–99.6%) when 600 EAU was the positive–negative cut-off determinant. The inter-assay inter-laboratory concordance of results using a 300 EAU or 600 EAU positive–negative cut-offs was demonstrated to be 97.8% and 98.8%, respectively.

Overall, no substantial difference among laboratory results was noted when evaluated using Deming correlation analysis. The average regression slope was calculated to be 1.029 (SD=0.121) and the average intercept was shown to be –24.115 (SD=38.204). To evaluate the strength of association with the magnitude of EAU results observed for each allergen among the different laboratories a Pearson's correlation coefficient was determined (Microsoft Excel 2007) for each laboratory

Table 2

Intra- and inter-laboratory concordance of results in relation to EAU cut-off.

Laboratory	Sample pairs N	Percent concordance at positive–negative cut-off		
		150 EAU	300 EAU	600 EAU
Intra-assay – proficiency run #1				
Agrolabo	360	96.9	98.6	98.6
Axiom	360	89.2	93.9	97.8
ARTU #1	324	91.7	97.5	99.4
Univet	360	94.7	97.8	99.4
Greer #1	360	96.1	98.6	98.1
Greer #2	360	97.2	97.8	98.9
Greer #3	288	95.5	96.2	97.6
Intra-assay – proficiency run #2				
Agrolabo	432	96.8	98.6	99.1
Axiom	432	94.7	96.8	99.5
ARTU #1	408	96.3	98.5	98.8
ARTU #2	408	96.1	99.3	98.8
Univet	432	94.0	98.4	99.8
BioVac	432	96.5	98.8	98.8
Greer #1	432	93.8	97.7	97.9
Greer #2	360	96.1	96.9	98.6
Greer #3	432	94.9	97.0	98.8
Greer #4	432	93.5	99.3	99.3
Inter-assay – intra-laboratory				
Agrolabo	792	96.8	98.6	98.9
Axiom	792	92.2	95.5	98.7
ARTU #1	732	94.3	98.1	99.1
ARTU #2	408	96.1	99.3	98.8
Univet	792	94.3	98.1	99.6
BioVac	432	96.5	98.8	98.8
Greer #1	792	94.8	98.1	98.0
Greer #2	720	96.7	97.4	98.8
Greer #3	432	94.9	97.0	98.8
Greer #4	720	94.3	98.1	98.6
Inter-assay – inter-laboratory				
	6612	95.0	97.8	98.8

Table 3Inter-laboratory correlation of Greer laboratories *macELISA* results and results observed in European affiliate laboratories.

Allergen	Inter-laboratory coefficient of correlation ^a				
	Greer/ARTU	Greer/Univet	Greer/Axiom	Greer/Agrolabo	Greer/BioVac
Mites					
<i>Acaris siro</i>	0.989	0.992	0.969	0.993	0.996
<i>Dermatophagoides farinae</i>	0.985	0.993	0.974	0.994	0.994
<i>Dermatophagoides pteronyssinus</i>	0.989	0.983	0.966	0.988	0.995
<i>Lepidoglyphus destructor</i>	0.985	0.980	0.972	0.954	0.979
<i>Tyrophagus putrescentiae</i>	0.993	0.994	0.967	0.994	0.987
Grasses					
June Grass (<i>Poa pratensis</i>)	0.988	0.994	0.970	0.981	0.995
Orchard Grass (<i>Dactylis glomerata</i>)	0.989	0.997	0.977	0.984	0.994
Meadow fescue (<i>Festuca pratensis</i>)	0.992	0.998	0.971	0.980	0.994
Perennial Rye (<i>Lolium perenne</i>)	0.989	0.997	0.978	0.989	0.995
Trees					
Birch (<i>Betula pendula</i>)	0.991	0.982	0.969	0.943	0.995
Cypress (<i>Cupressus sempervirens</i>)	0.996	0.989	0.980	0.968	0.997
Olive (<i>Olea europaea</i>)	0.992	0.995	0.993	0.981	0.998
Hazelnut (<i>Corylus avellana</i>)	0.995	0.992	0.977	0.955	0.993
Populus mix (<i>P. nigra</i> , <i>P. tremula</i> , <i>P. alba</i>)	0.994	0.994	0.974	0.968	0.995
Willow Black (<i>Salix discolor</i>)	0.996	0.994	0.981	0.958	0.995
Weeds					
Sheep Sorrel (<i>Rumex acetosella</i>)	0.993	0.994	0.979	0.986	0.996
Mugwort (<i>Artemisia vulgaris</i>)	0.989	0.992	0.966	0.986	0.996
Lambs Quarter (<i>Chenopodium album</i>)	0.988	0.992	0.978	0.981	0.987
English Plantain (<i>Plantago lanceolata</i>)	0.994	0.995	0.975	0.985	0.996
Ragweed (<i>Ambrosia trifida</i> , <i>A. artemisiifolia</i>)	0.993	0.997	0.984	0.989	0.989
Pellitory (<i>Parietaria officinalis</i>)	0.989	0.991	0.962	0.982	0.993
Fungi					
<i>Alternaria alternata</i>	0.328	0.198	0.161	0.316	0.586
<i>Aspergillus fumigatis</i>	0.592	0.778	0.140	0.771	0.844
<i>Cladosporium herbarum</i>	0.635	0.788	0.463	0.895	0.883

^a Pearson correlation coefficient (*r*); good correlation ($r > 0.9$, $p < 0.001$).

pair. The results presented in Table 3 demonstrate that high inter-laboratory correlation ($r > 0.90$; $p < 0.001$) is evident between the results observed in Greer's laboratory and those observed in all of the individual European affiliate laboratories for all mites and pollen allergens. Similarly there was high inter-laboratory correlation ($r > 0.90$; $p < 0.001$) between and among the various European laboratories (Table 4) for all mite and pollen allergens tested.

Good correlation with the fungal allergens was not evident within or between any of the testing laboratories. The magnitude of responses for the majority of responses for *Alternaria alternata* (519/560), *Aspergillus fumigatis* (467/560), and *Cladosporium herbarum* (443/560) was less than the positive–negative cut-off of 150 EAU. The magnitude of response for 246 of the remaining 251 responses was in excess of 150 EAU, but less than 300 EAU. Furthermore, the maximum positive signal evident with any of the fungi replicates was 370 EAU. Because of the extensive variability known to exist in this range of reactions, the responses evident with fungi were not included in the correlation comparison of responses within and between laboratories for the two separate runs.

The results presented in Table 5 compare the Pearson correlation evident within and between the two separate proficiency evaluations. Strong correlation ($r > 0.9$; $p < 0.001$) was evident with all allergens in all laboratories within each of the proficiency evaluations. The correlation between the two proficiency runs in all laboratories was also high for all allergens except olive

pollen. The poor correlation with olive between the two proficiency evaluations was traced to differing lot numbers of allergen extracts used in coating the wells. The average magnitude of responses (Table 6) evident with olive allergen differed by an average of nearly four-fold (range 1.69–11.89), but substantially different negative responses were not detected. Using a positive–negative cut-off of 150 EAU, 8 out of the 10 samples evaluated would have still yielded concordant results in spite of the dramatic differences in magnitude of response evident in the separate runs.

The final comparative evaluation for this initial proficiency monitoring of the various laboratories was an evaluation of the dose–response that is evident in *macELISA*. For this evaluation, a serial dilution of a positive pollen pan reactive sample was prepared using negative sera as diluent. Each of the dilutions was then evaluated in each of the participating laboratories as unknown independent samples. The comparative dose–response of *macELISA* observed with differing pollen allergens is presented in Fig. 1. Similar responses were yielded by all of the laboratories and the results observed within the various laboratories are encompassed by the acceptable variance limits (Lee et al., 2009) ($\pm 20\%$) established for *macELISA*. Further, the magnitude of responses observed in each laboratory was reduced in direct proportion to dilution. The dose–response curve that is observed in each laboratory approximates a second order polynomial function (i.e. quadratic), which is consistent with expected reactivity profile for assays of this type (Tijssen,

Table 4

Inter-laboratory correlation of macELISA results among affiliate laboratories.

Allergens	Inter-laboratory ^a coefficient of correlation									
	AR/UN	AR/AX	AR/AG	AR/BV	AG/UN	AG/AX	AG/BV	UN/AX	UN/BV	AX/BV
Mites										
<i>Acaris siro</i>	0.973	0.983	0.970	0.995	0.992	0.948	0.992	0.961	0.987	0.973
<i>Dermatophagoides farinae</i>	0.975	0.982	0.973	0.996	0.992	0.964	0.995	0.970	0.985	0.978
<i>Dermatophagoides pteronyssinus</i>	0.985	0.957	0.983	0.994	0.987	0.969	0.996	0.946	0.992	0.995
<i>Lepidoglyphus destructor</i>	0.971	0.963	0.966	0.958	0.980	0.945	0.990	0.971	0.989	0.974
<i>Tyrophagus putrescentiae</i>	0.986	0.962	0.989	0.986	0.995	0.953	0.988	0.955	0.985	0.972
Grasses										
June Grass (<i>Poa pratensis</i>)	0.985	0.979	0.974	0.995	0.982	0.950	0.991	0.964	0.991	0.962
Orchard Grass (<i>Dactylis glomerata</i>)	0.985	0.974	0.972	0.997	0.984	0.968	0.984	0.976	0.992	0.955
Meadow fescue (<i>Festuca pratensis</i>)	0.985	0.975	0.969	0.996	0.981	0.960	0.971	0.966	0.992	0.960
Perennial Rye (<i>Lolium perenne</i>)	0.985	0.980	0.985	0.998	0.987	0.975	0.994	0.974	0.994	0.965
Trees										
Birch (<i>Betula pendula</i>)	0.979	0.946	0.913	0.988	0.941	0.976	0.978	0.947	0.990	0.980
Cypress (<i>Cupressus sempervirens</i>)	0.985	0.981	0.967	0.995	0.986	0.988	0.988	0.983	0.988	0.989
Olive (<i>Olea europaea</i>)	0.985	0.988	0.980	0.998	0.988	0.986	0.994	0.992	0.994	0.995
Hazelnut (<i>Corylus avellana</i>)	0.988	0.979	0.958	0.990	0.955	0.986	0.962	0.973	0.993	0.972
Populus mix (<i>P. nigra</i> , <i>P. tremula</i> , <i>P. alba</i>)	0.987	0.970	0.962	0.994	0.980	0.976	0.985	0.982	0.997	0.970
Willow Black (<i>Salix discolor</i>)	0.993	0.979	0.955	0.995	0.970	0.963	0.983	0.981	0.996	0.972
Weeds										
Sheep Sorrel (<i>Rumex acetosella</i>)	0.990	0.982	0.982	0.998	0.992	0.985	0.990	0.988	0.996	0.978
Mugwort (<i>Artemisia vulgaris</i>)	0.985	0.962	0.975	0.989	0.990	0.982	0.984	0.977	0.992	0.982
Lambs Quarter (<i>Chenopodium album</i>)	0.984	0.976	0.980	0.995	0.986	0.990	0.984	0.978	0.989	0.990
English Plantain (<i>Plantago lanceolata</i>)	0.987	0.969	0.978	0.997	0.992	0.967	0.986	0.977	0.988	0.947
Ragweed (<i>Ambrosia trifida</i> , <i>A. artemisiifolia</i>)	0.991	0.982	0.986	0.990	0.991	0.978	0.981	0.983	0.988	0.983
Pellitory (<i>Parietaria officinalis</i>)	0.984	0.961	0.980	0.992	0.988	0.966	0.986	0.978	0.980	0.948
Fungi										
<i>Alternaria alternata</i>	0.200	−0.100	0.296	0.605	0.417	0.642	0.741	0.212	0.407	0.760
<i>Aspergillus fumigatus</i>	0.604	−0.066	0.466	0.823	0.607	0.358	0.844	0.080	0.707	0.526
<i>Cladosporium herbarum</i>	0.689	0.466	0.598	0.901	0.696	0.527	0.841	0.427	0.813	0.802

^a AR = ARTU; UN = Univet; AX = Axiom; BV = BioVac.

1993). The reaction profiles for grass allergens appear to be parallel and quite similar in magnitude of response. Although the responses evident to tree and weed allergens are more variable in magnitude of response, the

observed response in each laboratory was reduced in direct proportion to dilution. The positive response profiles evident with these allergens also appear to be parallel.

Table 5

Correlation of macELISA results within and between proficiency runs – effect of different lots of allergen coated wells on magnitude of response.

Allergen	Run #1	Run #2	Average runs 1 and 2	Run #1 vs run #2
Mites				
<i>Acaris siro</i>	0.993	0.994	0.986	0.995
<i>Dermatophagoides farinae</i>	0.991	0.995	0.986	0.995
<i>Dermatophagoides pteronyssinus</i>	0.964	0.994	0.972	0.974
<i>Lepidoglyphus destructor</i>	0.984	0.981	0.969	0.980
<i>Tyrophagus putrescentiae</i>	0.989	0.993	0.984	0.989
Grasses				
June Grass (<i>Poa pratensis</i>)	0.990	0.987	0.982	0.972
Orchard Grass (<i>Dactylis glomerata</i>)	0.994	0.987	0.986	0.990
Meadow fescue (<i>Festuca pratensis</i>)	0.991	0.986	0.984	0.987
Perennial Rye (<i>Lolium perenne</i>)	0.993	0.987	0.986	0.989
Trees				
Birch (<i>Betula pendula</i>)	0.980	0.990	0.972	0.973
Cypress (<i>Cupressus sempervirens</i>)	0.991	0.995	0.984	0.991
Olive (<i>Olea europaea</i>)	0.992	0.996	0.990	0.657
Hazelnut (<i>Corylus avellana</i>)	0.988	0.989	0.978	0.979
Populus mix (<i>P. nigra</i> , <i>P. tremula</i> , <i>P. alba</i>)	0.987	0.989	0.982	0.973
Willow Black (<i>Salix discolor</i>)	0.987	0.990	0.980	0.983
Weeds				
Sheep Sorrel (<i>Rumex acetosella</i>)	0.994	0.991	0.988	0.991
Mugwort (<i>Artemisia vulgaris</i>)	0.991	0.991	0.981	0.991
Lambs Quarter (<i>Chenopodium album</i>)	0.989	0.994	0.985	0.983
English Plantain (<i>Plantago lanceolata</i>)	0.995	0.987	0.987	0.987
Ragweed (<i>Ambrosia trifida</i> , <i>A. artemisiifolia</i>)	0.991	0.994	0.988	0.985
Pellitory (<i>Parietaria officinalis</i>)	0.980	0.983	0.979	0.992

Table 6

Comparative EAU responses observed with olive allergens during two separate proficiency evaluations.

Sample number	Run #1 EAU		Run #2 EAU		Run #1 EAU/Run #2 EAU ^b
	Average ^a	STD	Average ^a	STD	
10	2944	226	1741	171	1.69
1	2864	390	241	40	11.89
9	1524	133	705	95	2.16
8	558	44	266	47	2.10
3	455	102	113	22	4.03
7	198	45	115	33	1.72
4	99	50	75	27	1.33
2	36	41	30	18	1.19
6	30	24	35	15	0.85
5	18	16	19	15	0.96

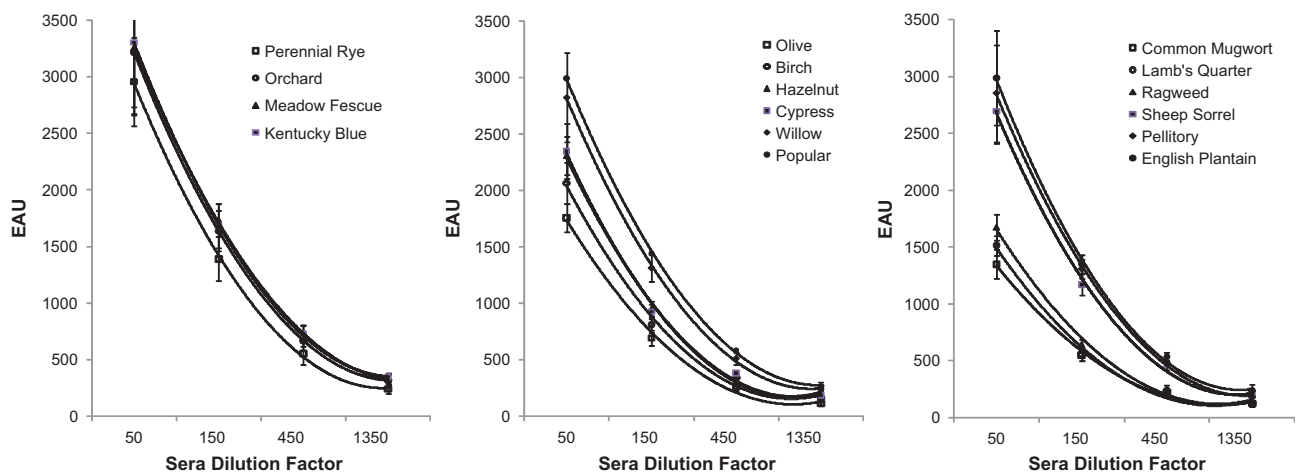
^a Average response of all participating laboratories.^b Ratio of responses observed with different lots of wells used in two different proficiency evaluations.

4. Discussion

The results of the present study demonstrate that the variability between and among the European laboratories is indistinguishable from the results evident within and between runs completed in Greer's laboratory. Although Greer developed and manufactures the assay components used in all laboratories, the results presented herein demonstrate that all laboratories are equally proficient in providing consistent results for all allergens tested. The intra-assay variance observed with the positive calibrators evident among the various runs within each of the laboratories remains relatively low and indistinguishable among the various laboratories. Likewise, the inter-assay variance within each laboratory remained relatively constant and the results from all laboratories were demonstrably similar and the CV of the positive responses was relatively constant across the entire range of reactivity tested. Consequently, the observed inter-laboratory CV was well within the acceptable variance limits ($\pm 20\%$) established for this assay (Lee et al., 2009). The variability of background and negative sera responses, in contrast, was substantially greater. The increased variability with the negative responses emphasizes the need to establish an

appropriate and robust cut-off for each assay. For the *macELISA*, a cut-off of 150 EAU establishes 99% confidence for positive responses.

Differentiating between positive and negative responses provides the basis of selecting allergen extract to be included in an immunotherapeutic regimen (Olivry and Sousa, 2001; Griffin and Hillier, 2001; DeBoer and Hillier, 2001b). Using 150 EAU as the positive–negative determinants, the overall inter-laboratory concordance of results was 95% which is consistent within and among laboratories as well as with previously defined observations. Increasing the cut-off to a greater level of certainly for detecting positive responses concomitantly increases the concordance of results observed in all laboratories. However, establishing an absolute cut-off at any level within a dynamic range of possible responses will undoubtedly lead to discrepant results, not only between, but within laboratories as well. Such will happen because of the variance that might occur at any given point in the response curve; even slight variances could result in discordant results. Thus, a better evaluation would be the correlation of results observed in and between laboratories. Using Pearson's correlation statistic, we have demonstrated excellent strength of association with magnitude of responses between and

**Fig. 1.** Overall dose–response evident with *macELISA*.

among all laboratories for all allergens except fungi. Lack of correlation with the responses evident with the fungal allergens is likely a consequence of responses for the vast majority falling within the negative range (<150 EAU) and no response to any fungi exceeded 370 EAU. Because the variance within this range of the response curve can be exceedingly high, the results do not lend themselves well to comparative evaluations and exemplifies the futility in evaluating the significance of differences in responses in this range of the response curve (Patterson et al., 2005).

There is no compelling evidence that the level of allergen specific IgE correlates with severity of clinical disease (DeBoer and Hillier, 2001b). However, an evaluation that purports to measure allergen specific IgE should have a reduction in signal that is directly proportional to the dilution factor of the test ligand (Tijssen, 1993). In assessing the dose–response curves evident in *macELISA* it becomes apparent that a three-fold dilution of serum results in an approximate two-fold reduction in signals generated. Thus, the relative amount of allergen specific IgE that might be detected at the cut-off level (150 EAU) will need to be increased three-fold to yield a response of 300 EAU and nine-fold to yield a response of 600 EAU; to generate a maximal signal (4000 EAU) will require nearly a 150-fold increase in allergen specific IgE. This being the case, it is unlikely that a highly reactive serum sample will be detected as non-reactive at a 1:5 dilution. The variance evident in 0–300 EAU range of responses dictates that true borderline positive samples might be identified as false negative responses and this tendency might compound the likelihood of false negative responses. However, a serum sample at a 1:5 dilution makes detection of false positive results seem rather remote.

Important in any longitudinal evaluation of ELISA responses is the inclusion of a comparison between differing lots of allergen coated wells. Within this study, two separate proficiency evaluations were completed using two different lots of allergen coated wells that were each prepared with different manufacture lots of allergen extracts. Each lot of wells was used with predefined quality control reactivity potential defined, but the reaction levels of the two lots were not (by design) compared or controlled. The average Pearson correlation coefficient among all allergens within the two proficiency runs was high, and strong correlation between runs was evident for all allergens except olive. The differences in responses to the olive allergens are clearly a result of differences in the two well lots. Whether the difference is merely a discrepancy in the coating of the two lots or a qualitative and/or quantitative difference in the allergen extracts themselves remains to be determined. None the less, these results emphasize the importance of adequately coated wells and institution of a quality assurance program to ensure reproducibility and uniformity of manufactured lots of wells. Equally important is the necessity for qualifying each new lot of allergen extract that is to be used in the well manufacturing process.

This report confirms that it is possible to establish and monitor intra- and inter-laboratory standardization to ensure the uniformity, consistency, and reproducibility of results reported among different laboratories that perform the same testing services. What is now needed is

establishment of an industry-wide voluntary quality assurance program for serum allergen-specific IgE testing that independently monitors performance of all laboratories and assay formats. The various parameters evaluated herein might serve as a prototype proficiency monitoring program among all veterinary laboratories. Until such an external and independent quality assurance program for testing of serum allergen-specific IgE is undertaken, individual laboratory studies of this sort combined with independent peer reviewed studies will be the only means of verifying the reliability of test results.

Source of funding

Funding for this study was provided by Greer Laboratories Inc.

Conflict of interest

All authors from Greer Laboratories are employees at Greer Laboratories Inc.; other authors are employees of the affiliate laboratories.

Acknowledgement

The authors thank Drs. Tom Grier and Terrance Coyne for their critical review of the content of this document.

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