Research Article

Proficiency Monitoring of Allergen-Specific IgE *mac*ELISA - 2022

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Abstract

The purpose of this study was to evaluate the reproducibility of results yielded using a *mac*ELISA for detection of llergen specific IgE in dogs and cats when run by ten different individuals in seven separate affiliated laboratories. Samples of 24 different sera samples were independently evaluated in each laboratory by differing operators in a single blinded fashion. For evaluations completed by multiple operators in a single laboratory, the average inter-operator variance was calculated to be 7.9% (range=6.5%-9.0%). The average intra-assay variance among reactive assay calibrators in all laboratories was 7.3% (range=2.8%-17.6%). The overall inter-assay inter-laboratory variance evident with reactive calibrators was consistent among laboratories and averaged 15.7% (range 14.8%-16.7%). All laboratories yielded similar profiles and magnitudes of responses for replicate unknown samples; dose response profiles observed in each of the laboratories were indistinguishable. Correlation of EAU observed for individual allergens between and among all laboratories was strong (r>0.85, p<0.001). Collectively, the results demonstrated that ELISA for measuring allergen specific IgE is reproducible, and documents that consistency of results can be achieved not only in an individual laboratory, but among different operators and between laboratories using the same ELISA.

Keywords: IgE, ELISA, Proficiency, Atopy, Allergy, Immunotherapy, Cross-reactive Carbohydrate

Introduction

Stallergenes Greer maintains a proficiency monitoring program for laboratories that routinely run *mac*ELISA [1] for evaluation of allergen specific IgE in serum samples. The foundation for this program is based on the desire for inter-laboratory standardization and quality control measures that ensure the uniformity, consistency, and reproducibility of results among laboratories that perform the assays. This program is designed to evaluate the proficiency of laboratories and ensures that individual operators yield consistent and reproducible results. The inaugural proficiency evaluations, initiated in 2009 and repeated in 2010, in six different laboratories documents that inter-laboratory standardization and quality control measures in the veterinary arena are on the immediate forefront and that uniformity, consistency, and reproducibility of results between laboratories is achievable [2]. Similarly, reproducibility of results among different laboratories was documented in the subsequent proficiency evaluations completed in 2013 [3], 2016 [4], 2018 [5], 2019 [6], 2020 [7], and 2021 [8]. The results presented herein summarize the comparative results observed in the affiliate laboratories for the most recent proficiency evaluations that were completed in 2022. The 2022 proficiency evaluation is the fourth documentation of the assay reproducibility since adopting a cross-reactive carbohydrate inhibitor in the sample diluent [6-9].

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 Stallergenes 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.

Participating Laboratories

Seven independent Veterinary Reference Laboratories (VRLs) participated in the 2022 proficiency evaluation of *mac*ELISA. Participating laboratories included three separate IDEXX laboratories located in Memphis, Tennessee, Kornwestheim, Germany, and Markham, Ontario Canada. Other affiliated European laboratories that participated in this evaluation included Agrolabo (Scarmagno, Italy), Laboratories LETI Pharma (Barcelona, Spain), and Ceva Biovac (Beaucouzé, France). Stallergenes Greer Laboratories (Lenoir, NC) served as the prototype for evaluation of the *mac*ELISA; the 2022 evaluations included results reported by four separate and independent operators. Because the performance characteristics of *mac*ELISA in Stallergenes Greer's VRL have been well documented for use over an extended period [1-8], all results observed in the other

participating laboratories were compared directly with the results observed in Stallergenes Greer's reference laboratory.

Serum Samples

Separate pollen and mite reactive serum pools or non-reactive sera pools were prepared from cat and dog serum samples that previously had been evaluated using the *mac*ELISA for detection of allergen specific IgE. The reactivity of each sera pool ranged from nonreactive to reactive for multiple pollen or mite allergens. 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. Twenty-four samples were included in the blinded evaluation conducted by each laboratory. Identical replicates of the high, low, and negative controls routinely used in the assay were also included as unknown samples. Also included in the array of samples was a five tube three-fold serial dilution of a highly pollen reactive pool, diluted into non-reactive sera, which served to document the dose response evident within the assay. All samples were stored at -20 °C for the interim between testing.

Calibrators

Mite reactive calibrator solutions of predetermined reactivity in the *mac*ELISA were prepared as three-fold serial dilutions of a sera pool highly reactive to *Dermatophagoides farinae, Acarus siro,* and *Tyrophagus putrescentiae*. Replicates of each were evaluated in each assay run and served as a standard response curve for normalizing results observed with the various samples. All results were expressed as ELISA Absorbance Units (EAU) which are background-corrected observed responses expressed as milli absorbance.

Buffers

The buffers used throughout have been previously described [1-8], 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) reagent diluent buffer: PBS, pH 7.4, containing 1% fish gelatin, 0.05% Tween 20 and 0.05% sodium azide. The buffer used for dilution of serum samples was identical to the reagent diluent buffer, but it has been supplemented (2.5 mG/mL) with a cross-reactive carbohydrate inhibitor derived from the carbohydrate components present in bromelain (BROM-CCD) [9]. BROM-CCD was prepared in house and remains a proprietary product of Stallergenes Greer (Lenoir, NC, USA).

Allergen Panel

The allergen panel was a 24 allergen composite derived from the array of allergens that are included in the specific panels routinely evaluated in the various laboratories; the composite allergen panel consisted of 4 grasses, 6 weeds, 6 trees, 5 mites, and 3 fungi. The protocol for coating and storage of wells has been previously described [1-8]. Immulon 4HBH flat bottom 12 well strip assemblies (Thermo Electron Corporation, Waltham, MA) were used throughout and served as the solid phase for all assays. The individual extracts were diluted in bicarbonate buffer (pH 9.6) and 100 µL 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.

Sample Evaluations - *mac*ELISA

The operational characteristics and procedures for the *mac*ELISAs have been previously described [1-8]. Following incubation of allergen coated wells with an appropriately diluted serum sample, allergen-specific IgE is detected using a secondary antibody mixture of biotinylated monoclonal anti-IgE antibodies, streptavidin alkaline phosphatase as the enzyme conjugate, and *p*-nitrophenylphosphate (*p*NPP) as substrate reagent. Specific IgE reactivity to the allergens is then estimated by determining the absorbance of each well measured at 405 nM using an automated plate reader. All results are expressed as ELISA Absorbance Units (EAU), which are background-corrected observed responses expressed as milli absorbance [1].

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. Pearson's correlation statistic was used for inter-laboratory comparison among individual allergens. Statistical analyses were conducted using EXCEL (2016; Microsoft; Redmond, WA, USA).

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 (#1-5) was 7.3% (range=2.8%-17.6%); differences among laboratories or between assays and within assay runs were not detected. No substantial difference in results among various operators were revealed. The average inter-operator variance documented for Stallergenes Greer technicians was calculated to be 7.9% (range=6.5%-9.0%). Increased intra-assay variability was evident with the background ODs (average 9.2%; range 4.8%-22.5%). A negative response is classified as anything with an EAU below 150 [1]. Any analysis of results below this threshold, especially when looking at %CV and relative differences, should be done cautiously.

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 2016) for each laboratory pair. Because the *mac*ELISA is designed to yield comparable responses in dog and cat samples, comparison of results among affiliate laboratories included both cat and dog samples as a single population of sera samples [5-8]. These results (Table 2) demonstrate that good inter-laboratory correlation (r>0.80; p<0.001) is evident between the results observed in Stallergenes Greer laboratory and those observed in six affiliate laboratories for all pollen, mite, and fungi allergens. The overall correlation of results observed in the various laboratories are summarized in Table 3; a very strong correlation (r>0.90, p<0.001) was demonstrated between and among the results of the participating laboratories.

Table 1: Calculated variance of *mac*ELISA calibrator solutions observed with different laboratory runs by multiple operators during the 2021 Proficiency evaluation.

Variance	N	Calibrator % CV*					BG†
		#1	#2	#3	#4	#5	% CV
Inter-Laboratory	320	14.8	16.7	16.4	14.9	15.1	26.1
Inter-Assay (Stallergenes Greer)	128	7.4	9.0	8.6	6.5	5.9	8.2
Intra-Assay							
Stallergenes Greer #1	32	6.1	6.2	6.2	4.4	3.4	5.4
Stallergenes Greer #2	32	7.6	8.4	9.5	7.2	6.2	9.5
Stallergenes Greer #3	32	4.4	4.1	4.9	4.8	4.5	6.6
Stallergenes Greer #4	32	2.8	4.1	3.9	3.8	4.8	4.8
IDEXX Memphis	32	13.0	14.8	17.6	12.4	6.6	5.7
IDEXX Canada	32	2.8	4.5	7.0	6.0	10.8	22.5
IDEXX Germany	32	8.1	13.2	6.4	4.2	4.5	9.4
Agrolabo	32	7.0	8.0	6.7	6.1	10.3	12.4
Biovac	32	10.5	14.4	7.2	6.3	7.9	5.7
LETI	32	7.2	9.3	6.0	5.5	12.7	10.2

*Calibrator #1 is prepared as a dilution of a sera pool which is highly reactive to mite allergens; Calibrators #2 - #5 are prepared as a serial 3-fold dilution of calibrator #1.
†Background responses observed with diluent in place of serum sample.

Table 2: Inter-laboratory correlation of *mac*ELISA results observed with individual allergens in Stallergenes Greer Laboratory and the results observed in the individual affiliate laboratories.

Allergens	Inter-Laboratory Coefficient of Correlation* Stallergenes Greer vs					
	IDEXX Memphis	IDEXX Germany	IDEXX Canada	Ceva Biovac	Agrolabo	LETI
Mites						
Acaris siro	0.952	0.997	0.987	0.980	0.975	0.981
Dermatophagoides farinae	0.903	0.964	0.954	0.938	0.925	0.926
Dermatophagoides pteronyssinus	0.972	0.987	0.968	0.979	0.923	0.923
Lepidoglyphus destructor	0.962	0.986	0.958	0.974	0.862	0.888
Tyrophagus putrescentiae	0.967	0.994	0.988	0.946	0.975	0.982
Grasses						
June Grass (*Poa pratensis*)	0.957	0.990	0.993	0.968	0.959	0.961
Meadow fescue (*Festuca pratensis*)	0.937	0.980	0.989	0.952	0.941	0.941
Orchard Grass (*Dactylis glomerata*)	0.943	0.984	0.990	0.957	0.947	0.946
Perennial Rye (*Lolium perenne*)	0.942	0.981	0.992	0.960	0.950	0.946
Trees						
Birch (*Betula pendula*)	0.946	0.986	0.978	0.953	0.945	0.946
Cypress (*Cupressus sempervirens*)	0.943	0.941	0.949	0.932	0.902	0.952
Hazelnut (*Corylus avellana*)	0.970	0.985	0.981	0.957	0.963	0.952
Olive (*Olea europaea*)	0.943	0.985	0.981	0.959	0.947	0.943
Populus mix (*P. nigra, P. tremula, P. alba*)	0.944	0.974	0.969	0.942	0.941	0.932
Willow Black (*Salix discolor*)	0.961	0.986	0.961	0.959	0.954	0.954
Weeds						
English Plantain (*Plantago lanceolata*)	0.956	0.988	0.977	0.965	0.942	0.939
Lambs Quarter (*Chenopodium album*)	0.957	0.931	0.886	0.952	0.805	0.905
Mugwort (*Artemisia vulgaris*)	0.953	0.978	0.972	0.952	0.949	0.920
Pellitory (*Parietaria officinalis*)	0.960	0.981	0.972	0.963	0.959	0.933
Ragweed (*Ambrosia trifida, A. artemisiifolia*)	0.995	0.996	0.999	0.999	0.998	0.999
Sheep Sorrel (*Rumex acetosella*)	0.935	0.996	0.982	0.963	0.945	0.963
Fungi						
Alternaria alternata	0.918	0.992	0.965	0.938	0.879	0.930
Aspergillus fumigatis	0.983	0.995	0.999	0.993	0.976	0.992
Cladosporium herbarum	0.981	0.976	0.968	0.979	0.915	0.952
Overall	0.936	0.983	0.973	0.949	0.947	0.961

*Pearson Correlation Coefficient (r); Good Correlation (r > 0.8, p<0.001).

Table 3: Inter-laboratory correlation of *mac*ELISA results observed among individual affiliate laboratories.

Laboratory	Interlaboratory Coefficient of Correlation*						
	Stallergenes Greer	IDEXX Memphis	IDEXX Germany	IDEXX Canada	Ceva Biovac	Agrolabo	LETI
Stallergenes Greer	1	0.936	0.983	0.973	0.949	0.947	0.961
IDEXX Memphis	0.936	1	0.961	0.964	0.964	0.960	0.965
IDEXX Germany	0.983	0.961	1	0.987	0.970	0.967	0.981
IDEXX Canada	0.973	0.964	0.987	1	0.982	0.980	0.984
Biovac	0.949	0.964	0.970	0.982	1	0.986	0.982
Agrolabo	0.947	0.960	0.967	0.980	0.986	1	0.987
LETI	0.961	0.965	0.981	0.984	0.982	0.987	1

*Pearson Correlation Coefficient (r); Good Correlation (r > 0.8, p<0.001).

For an evaluation of the dose response in this ELISA, a five tube three-fold serial dilution of a reactive dog sera pool was prepared using a negative cat sera pool as diluent. Each of the dilutions was then evaluated by all of the participating laboratories as unknown independent samples. Similar responses were yielded by all of the laboratories and the results observed within the various laboratories are encompassed by the acceptable variance limits [1-3] (±20%) established for *mac*ELISA. Further, the magnitude of responses observed in each laboratory was reduced in direct proportion to dilution. Consequently, the dose responses for the individual allergens are presented as aggregate responses. The results presented in Figure 1 confirm the sera pool was highly reactive to mites as well as grass, weed, and tree pollen allergens, but it was not reactive to fungi. To be expected, the magnitude of responses observed in each laboratory was reduced in direct proportion to dilution. Results from the final tube in the dilution scheme yielded results that were indistinguishable from negative responses, indicating a dilution extinction of detectable response [9-14].

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