

Reproducibility of a commercial in vitro allergen-specific assay for immunoglobulin E in dogs

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Samples of serum taken from 42 dogs with clinical signs and histories indicating pruritic skin disease and/or diseases of the ear were tested in a commercial allergen-specific assay for immunoglobulin E. Dogs which had been treated with glucocorticoids and/or antihistamines were not excluded. The samples were separated into two equal aliquots, given different randomised numbers, and analysed in two batches on two separate days. The laboratory was blinded to the identification numbers and the history of each dog, but knew the purpose of the study. The results for 48 allergens were expressed in modified absorbance units (MAU). The overall median MAU was 29. For each allergen the mean difference between the MAU values of the paired duplicates was determined and the difference was compared to zero by a paired *t* test. The number of means that were not 0 ($P < 0.05$) in each allergen group were: seven of 10 grasses, seven of nine weeds, two of 13 trees, six of 10 fungi, and three of six environmental allergens. A single 2 x 2 table for the 48 allergens was created with MAU ≥ 60 defined as 'positive' and < 60 as 'negative'. There were 116 of 188 (62 per cent) pairs that were reproducibly 'positive' and 1756 of 1828 (96 per cent) pairs that were reproducibly 'negative'.

IN VITRO serum allergen-specific assays for immunoglobulin E (IgE), which were first described for use in dogs by Halliwell and Kunkle (1978), are commonly used by veterinary clinicians. They evaluate the specific levels of IgE against a panel of regional allergens that could be contributing to the dog's disease, and in combination with the dog's history and the results of a physical examination, they may help not only in the diagnosis of allergic skin disease (Scott and others 2001, Hamilton and Adkinson 2003), but also in determining the degree of sensitisation, and in identifying allergens for avoidance and treatment strategies (Szeinbach and others 2001). However, mistakes in diagnosis based on the use of these serological tests could result in treatments that may not be warranted.

Many commercial laboratories apply the assays to measure allergen-specific IgE antibodies in serum samples from dogs, but they are used without any external regulatory review. As a result the assays are not calibrated against an international standard and quality assurance programmes are implemented at the discretion of the laboratory (DeBoer and Hillier 2001). Veterinary clinicians therefore have to trust the laboratory to provide a dependable test.

The Federal Clinical Laboratory Improvement Act of 1988 (CLIA-88) requires all federally licensed medical laboratories in the USA performing allergy testing to take part in an external proficiency survey, sponsored by the College of American Pathologists (Anon 2002). Replicate samples of serum containing known amounts of human IgE are sent to participating laboratories for the analysis of total serum IgE, and variations between the results are compared, tabulated and sent to the participants and certifying agencies. If the total serum IgE level is out of the participants' group range (mean ± 3 sd), the laboratory is at risk of losing its federal licence for the test (Hamilton and Adkinson 2003). The use of the World Health Organization's IgE International Reference Preparation for calibrating different testing methods has improved interlaboratory agreement, and for serum IgE levels 30 kiu/l or more the variation between methods is less than 10 per cent (Hamilton and Adkinson 2003). Measurements of serum allergen-specific IgE are included in the survey, although they are not included in the CLIA-88 statutes; however, individual laboratories are under pressure to improve and maintain reliable tests to remain competitive. Nevertheless, the analytical results of several serological assays have

been shown to be substandard (Williams and others 2000, Szeinbach and others 2001).

Values for the sensitivity and specificity of in vitro allergen-specific tests for IgE in dogs have been reported (Codner and Lessard 1993, Miller and others 1993, Bond and others 1994, Hämmerling and de Weck 1998, Wassom and Grieve 1998, Zunic 1998, Mueller and others 1999), and overall these independent studies have concluded that they are neither 100 per cent sensitive nor 100 per cent specific when compared with the nearest gold standard available, intradermal testing. Nevertheless, no peer-reviewed studies on the reproducibility of these in vitro IgE assays have been reported; the objective of this study was therefore to determine the between-day reproducibility of one particular assay in duplicate serum samples from dogs.

MATERIALS AND METHODS

The study was approved by the University of Illinois Institutional Animal Care and Use Committee. Forty-two privately owned dogs from the Midwest with a history compatible with chronic allergic skin and/or ear disease, for example, seasonal or non-seasonal pruritus and/or recurring bacterial and/or yeast infections, which had been referred to the university dermatology service or a nearby private dermatology practice between August and November 2004 were used. There were three intact males, 24 neutered males, one intact female and 14 spayed females, ranging in age from nine months to 13 years (mean age 4.3 years). There were 13 mixed-breed dogs, five labrador retrievers, four English bulldogs, four cocker spaniels, three golden retrievers, and one each of American bulldog, pug, toy poodle, West Highland white terrier, dachshund, standard schnauzer, shar pei, Bernese mountain dogs, German shepherd dog, great dane, Saint Bernard, Portuguese water dog and weimaraner. Each of them had at least one sign of allergic skin and/or ear disease when they were enrolled, and current treatment with systemic and/or topical antibiotics, antihistamines and/or fatty acid supplements did not exclude them. Preliminary evidence suggests that glucocorticoids given at moderate doses do not affect the results of in vitro serum allergy tests in dogs (Miller and others 1992); there were 21 dogs not receiving oral glucocorticoids or antihistamines, 12 dogs receiving only anti-

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histamines, six dogs receiving only glucocorticoids, two dogs receiving both glucocorticoids and antihistamines and one dog whose previous treatment for pruritus was unknown.

Approximately 17 ml of whole blood was collected into tubes from a jugular or cephalic vein, allowed to clot, and then centrifuged at 1000 g for 10 minutes. The serum was aspirated into plastic vials which were identified with a number and stored at -70°C . After all the dogs had been sampled the samples were thawed to room temperature and each vial was inverted several times. The serum from each dog was divided into two equal aliquots and placed in plastic vials containing at least 1 ml per vial. The duplicates were then given separate randomised identification numbers.

The duplicate aliquots from each dog were separated into two batches, A and B, so that one duplicate from each dog was in each batch. Batch A was sent at room temperature by surface mail to Greer Laboratories (Lenoir, NC, USA), and batch B was sent in the same way two days later, after having been stored frozen at -70°C . Each aliquot was tested against 48 allergens common to the Midwest of the USA. The samples had been collected and submitted according to the laboratory's guidelines and the laboratory was aware of the purpose of the study. However, it agreed to analyse each batch on separate days, while being unaware of the history and identity of each sample, and it also agreed to provide quality assurance data and proprietary information not usually available to clients.

The laboratory uses an alkaline phosphatase ELISA for the semi-quantitative detection of allergen-specific IgE. The assay incorporates regional allergens, adsorbed on to the surface of polystyrene microtitre wells, that react with corresponding allergen-specific IgE if it is present in the serum. Any unbound antibodies are removed by a washing step, and a mixture of biotin-conjugated monoclonal anti-canine IgE detecting antibodies is then added to each well. The assay kit claims that other antibody isotypes, for example, IgG, are not detected by the monoclonal antibodies. After another washing step, the enzyme streptavidin alkaline phosphatase is added to each well and links to any detecting antibody bound in the well. After a final washing step, an enzyme-specific chromogenic substrate solution is added to the wells. The intensity of the coloured product in each well, measured photometrically at 405 nm, is proportional to the amount of allergen-specific IgE present in the serum. The optical densities (OD) are corrected for the background reading in wells without serum, multiplied by 1000, and reported as ELISA absorbance units (EAU), with a range from 0 to 4000. The level of allergen-specific IgE is estimated by comparison with the colorimetric response of five assay calibrators, which contain canine serum of known allergen reactivity (proprietary information). The allergen-specific IgE detected by the assay is reported to the clinician in modified absorbance units (MAU), by using a proprietary conversion factor from EAU, with a range from 0 to 245. The results are to be interpreted in combination with the clinical history and environment of the dog. Greer Laboratories suggests that MAU scores of 60 or above are positive, scores of 40 to 59 are equivocal, and scores of 0 to 39 are questionable. All the data and calculations were reported to the investigators as Excel (Microsoft Office 2003) spreadsheets. The EAU scores, calculations used to convert raw absorbance values to EAU, and other proprietary data were also reported.

Statistical analysis

An analysis of covariance was applied to the calibration data for batch A and batch B (proprietary data supplied by Greer Laboratories). For each allergen, the mean of the differences between the samples in batch A and batch B was tested against the expected mean of zero, by using a paired *t* test; the number of significant tests per group of allergens (grasses, weeds, trees, fungi and environmental) was determined. For each

TABLE 1: Results of duplicate paired modified absorbance units (MAU) differences (batch A – batch B) for each allergen associated with 10 grasses, nine weeds, 13 trees, 10 fungi and six environmental allergens

Allergen	Observed mean of paired differences	Largest absolute difference among duplicate aliquots	P
Grasses			
Bermuda	2.81	139.89	0.57
Corn pollen	7.04	52.14	0.03*
Johnson	10.97	47.28	0*
Kentucky blue/June	8.43	47.63	0.02*
Meadow fescue	6.55	44.88	0.04*
Orchard	6.72	51.23	0.06
Perennial rye	5.10	45.93	0.14
Quack	7.76	50.20	0.01*
Timothy	9.74	42.17	0*
Wheat pollen	11.76	40.49	0*
Weeds			
Cocklebur	9.48	128.60	0.03*
Dandelion	5.95	66.06	0.07
Dock/sorrel	8.03	40.93	0*
English plantain	11.40	55.98	0*
Goldenrod	11.15	75.60	0.01*
Kochia, firebush	12.62	50.36	0*
Lamb's quarter	14.46	56.40	0*
Pigweed	6.61	50.19	0.03*
Ragweed	6.06	52.23	0.05
Trees			
Ash	11.69	85.00	0.02*
Birch	1.68	53.50	0.65
Cedar, red	-3.32	62.32	0.33
Cottonwood	-2.93	55.36	0.52
Elm	6.19	50.54	0.12
Hackberry	-6.65	59.96	0.06
Hickory/pecan	-4.45	54.48	0.28
Maple	28.63	135.90	0*
Mulberry, red	0.55	48.87	0.87
Oak mix	2.80	51.61	0.42
Pine	3.59	40.08	0.19
Sycamore, eastern	1.27	144.70	0.83
Willow, black	-3.47	39.19	0.30
Fungi			
<i>Alternaria</i>	-0.34	34.39	0.87
<i>Aspergillus</i>	14.48	50.89	0*
<i>Cephalosporium</i>	-0.91	31.39	0.64
<i>Cladosporium</i>	6.31	41.22	0.03*
<i>Curvularia</i>	8.15	45.07	0*
<i>Malassezia</i>	5.51	48.45	0.05
<i>Mucor</i>	12.72	51.81	0*
<i>Penicillium</i>	13.12	43.95	0*
<i>Pullularia</i>	15.19	43.08	0*
<i>Stemphylium</i>	1.55	44.50	0.52
Environmental			
Cat	7.91	55.83	0*
<i>Dermatophagoides farinae</i> [†]	7.15	89.67	0.12
<i>Dermatophagoides pteronyssinus</i> [†]	5.52	39.77	0.07
Flea	14.63	62.21	0*
Human dander	4.09	59.92	0.06
<i>Tyrophagus putrescentiae</i> [‡]	13.50	45.94	0*

* Significant at $P < 0.05$

[†] House dust mites

[‡] Storage mite

allergen, the frequencies of negative and positive duplicate paired MAU differences (batch A – batch B) were analysed by using a sign test. The same analysis was applied to the EAU values and the proprietary cut-off values that corresponded to the MAU cut-off values. The observed binomial proportion of positive differences to total pairs was tested against the expected proportion of 0.5. Defining MAU ≥ 60 as 'positive' and < 60 as 'negative', the 2×2 table for batch A versus batch B was analysed by using Fisher's exact test, a conditional test for testing the equality of two binomial proportions. The tests of contingency tables used StatXact version 6 (Cytel), and the other statistical analyses used Systat version 11.0 software (Systat). A *P* value of < 0.05 was considered significant, and a *P* value of $0.05 < P \leq 0.1$ was considered 'marginally significant' or indicative of a 'trend'.

TABLE 2: Combined results of batch A tests matched paired to batch B tests across all allergens if modified absorbance units (MAU) ≥ 60 is considered positive and MAU < 60 is considered negative

		Batch B		Total
		Positive	Negative	
Batch A	Positive	116	25	141
	Negative	47	1756	1803
	Total	163	1781	1944

RESULTS

The results have been expressed in MAU because these are the values reported by the laboratory. Similar results were obtained by using the data reported in EAU, but the proprietary nature of these data prevents the differences between the analyses of the data expressed as MAU and EAU from being discussed.

Quantitative analysis

The MAU results for 12 allergens (birch, corn pollen, cottonwood, elm, hackberry, hickory/pecan, kochia or firebush, orchard, eastern sycamore, timothy, wheat pollen and black willow) for six samples in batch B (dogs 9, 10, 19, 20, 35 and 36) were lost in processing; as a result there were only 1944 pairs of samples rather than 2016 pairs. The median MAU was 29 and the mean was 32 (range 0 to 226) with a 95 per cent confidence interval from 31 to 33. The coefficient of variation for these samples was 90 per cent. Of the 3888 analyses, only 71 MAU values were over 150 (roughly the MAU value of the highest calibration standard) and 3246 were below the lowest calibration standard.

Theoretically, if the assay is reproducible, the mean of the paired differences for each allergen should be zero. Table 1 shows that none of the observed means of the duplicate paired differences for each allergen was zero, and that there were large differences between some duplicate pairs. The largest absolute difference between duplicate pairs was 145 for the eastern sycamore followed by 140 for Bermuda grass. Twenty-five of the possible 48 means of paired differences of MAU values were significantly different from zero, the proportion differing among the allergen groups: seven of 10 grasses, seven of nine weeds, two of 13 trees, six of 10 fungi and three of six environmental allergens. In each case the mean of batch A was greater than the mean of batch B. For the 23 allergens with mean differences that were not significantly different from zero, 16 were over 0 and seven were less than 0, that is the mean MAU of batch B was greater than that of batch A. An exact one-sided test against the expected proportion, 0.5, was significant.

Qualitative analysis

Only the duplicate pairs with MAU results for each allergen were included in the analysis. Out of 1944 allergen pairs, 592 had a mean difference (batch A – batch B) less than zero, 1196 differences were greater than zero, and 156 were zero because both MAU values were zero. Excluding these 156 pairs, there were 592 of 1788 differences less than zero (33 per cent) and 1196 of 1788 greater than zero (67 per cent); this distribution

differed significantly from the expected proportion of 0.5.

Of the 1944 allergen pairs, 116 were both positive, 1756 were both negative, and 72 were discordant (Table 2). At least one value in a pair was 'positive' for 188 pairs, and in 32 of these (17 per cent), both values were 150 or more. In batch A, 141 of 188 tests (75 per cent) were positive and in batch B 163 (87 per cent) were positive. The probability that a serum sample that was positive in one test was positive in both tests was 0.62 (116/188). Of the 1828 pairs in which at least one MAU value was classified as 'negative', 1756 (96 per cent) were negative in both tests. For the discordant duplicate pairs, more than half of the duplicate tests were less than 20 per cent below the cut-off MAU value of 60 (Table 3).

Calibration analysis

The analysis of covariance carried out on the confidential calibration data for batch A and batch B showed that there was a significant difference between the slopes of the calibration lines ($P=0.042$).

DISCUSSION

There were significant differences, both quantitatively and qualitatively, between the MAU scores recorded from duplicate samples of serum from dogs with a history and clinical signs compatible with allergic skin and/or ear disease analysed on two separate days. For this sample of dogs the reproducibility of the assay for IgE, and particularly the ability to make a clinical determination of significance, was marginal.

Serological tests for allergy have several advantages over intradermal tests, including convenience, minimal risk, little need for the withdrawal of antipruritic pharmacotherapy before the test, and no need to interpret reactions on already inflamed skin (Scott and others 2001). However, for these advantages to be beneficial, any serological test must be accurate and reproducible. The sensitivity and specificity of various in vitro allergen-specific IgE assays for canine allergies have been described by Codner and Lessard (1993), Miller and others (1993), Bond and others (1994), Hämmerling and de Weck (1998), Wassom and Grieve (1998), Zunic (1998), and Mueller and others (1990), but none of them was either very sensitive or specific in comparison with the gold standard of intradermal testing, which itself may not be reproducible. There are no recognised allergen standards against which veterinary laboratories can calibrate their assays, and no standardised source of allergen extracts, and as a result many different methods are used to measure allergen-specific IgE, for example, preassay serum treatment, solid versus liquid phase IgE capturing techniques, anti-canine IgE versus high affinity IgE receptor ($FC\epsilon R1\alpha$) detecting reagents, and colorimetric versus radiometric methods; furthermore there is no standardised reporting scheme, and no external regulatory agency overseeing quality assurance procedures; therefore, it is not possible to compare the results obtained by different laboratories on the same sample (DeBoer and Hillier 2001). As a result veterinary clinicians are responsible for validating a laboratory's assay results against the animal's clinical data, unlike their physician counterparts who can investigate companies so as to send blood samples for IgE testing to laboratories that are CLIA-88 certified, perform well in the diagnostic allergy proficiency survey, and exercise validated methods (Hamilton 2001, Hamilton and Adkinson 2003).

The reproducibility of the assay described here was evaluated quantitatively and qualitatively. The MAU score for a particular allergen in the same blood sample should be repeatable on different days, that is, any difference for one sample should be close to zero and the mean difference for a large number of samples should not be significantly differ-

TABLE 3: Frequency distribution of the percentage difference by which the modified absorbance units (MAU) value was below 60 for the 72 discordant duplicate analyses (one analysis ≥ 60 MAU and other < 60 MAU for the same allergen)

	Percentage difference										Total
	-100	-90	-70	-60	-50	-40	-30	-20	-10	0	
Number of duplicates	3	3	6	5	5	5	4	3	22	16	72

ent from zero. Across the 48 allergens, 52 per cent of the mean differences were significantly different from zero. The MAU results for tree allergens were the most reproducible, with only ash and maple having means that differed significantly. Only 304 of the 3888 paired allergen tests gave 'positive' results, leaving 3584 'negative' results. This was not surprising because the mean MAU score was only 32. The negative MAU scores were reproducibly negative (96 per cent), but the positive scores had a reproducibility of only 62 per cent. There were 72 results which could have changed a clinical treatment if taken out of context with the animal's history and clinical signs; in three of these the MAU was reported as zero in one batch and 60 or more in the other.

Plant (1994) recorded the reproducibility of three in vitro allergy tests for dogs, carried out by the reference laboratory used in this study. Duplicate aliquots of serum from three dogs with atopic dermatitis were sent to the laboratory on the same day, and 22 allergens were analysed. Of the 66 pairs of MAU values 14 (21 per cent) were positively concordant, 46 (70 per cent) were negatively concordant, and six (9 per cent) were discordant, on the basis of the scoring system recommended by the laboratory. Because the present study analysed allergens common to the Midwest, used a larger number of allergens, included more dogs not specifically identified as atopic, and focused on the interday reproducibility of the assay, direct comparisons between these two studies are not possible.

Murray and others (1993) categorised the variability in test results as either biological (intra- and interindividual) or non-biological (preanalytical and analytical). Intraindividual biological variation could be due to a stress response during sample collection. In the present study the duplicate samples were taken by a single venepuncture, and this source of variation was therefore not a factor. Interindividual variation was to be expected owing to the variety of breeds of dog of different ages, weights, and medication histories, and potentially different underlying dermatoses, for example, flea hypersensitivity, food hypersensitivity, atopic dermatitis and sarcopic mange. However, because the study was examining the reproducibility and not the accuracy of the assay, the cause of the dermatitis was irrelevant. The histories and clinical signs of the dogs were suggestive of allergies, but the low MAU values for most of the analyses suggested that not many of the dogs were atopic; however, the low MAU scores could have been due either to previous antipruritic treatment or to the presence of dogs in which IgE did not play a significant role in their disease, or to errors in the assay.

Preanalytical variation could have included the mislabeling of samples, and incorrect preliminary processing, storage and/or shipment (Murray and others 1993). No mistakes in labeling were detected, and the samples were processed according to the laboratory's guidelines. The effect of the additional freeze/thaw cycles for batch B should have been negligible, because Hill and DeBoer (1994) reported minimal changes in serum IgE concentration after 25 freeze/thaw cycles. Analytical variations can be due to sample mix-up or mishandling, incorrectly calibrated or malfunctioning instruments, out-of-date reagents, inappropriate timing or temperature for the assay procedure, failure to follow the assay protocol strictly, and a lack of quality assurance procedures (Murray and others 1993). The reagents used for batch A and batch B had identical lot numbers, and the calibrator standards gave the expected EAU values. For quality assurance purposes, the laboratory uses internal high, low and negative control serum samples, which are analysed in parallel with the samples of interest. The laboratory did not report any discrepancies in its control readings, and no discrepancies were detected in the analyses of the data from the calibration standards. Since the laboratory markets the assay as a semi-quantitative IgE detection system, the serum IgE is expressed

in terms of MAU scores based on comparisons with intra-assay calibrator readings.

The laboratory computes a normalisation factor as the average of all the calibration replications as the ratio of 'expected' OD/'observed' OD, where the expected OD value is determined by the manufacturer of the kit used in the analysis. This normalisation factor is the slope of a linear regression through the origin. The analysis of covariance applied to the calibration data for batch A and batch B did reveal a difference between the slopes of the calibration lines, and as a result the use of a normalisation factor could have led to different conclusions about clinical significance in the region around MAU 60. This may account for some of the disparities. However, some of the differences were very large, and must be ascribable either to the analysis, or to differences between the aliquots, or both, or to errors in the company's calculations. Errors in the calculations were probably not responsible because selected discrepant and concordant values could be verified. It must therefore be concluded that the results of this assay for serum allergen-specific IgE may not be reproducible. However, most of the samples had low levels of IgE and the reproducibility of the assay may be better at the higher levels. Some evidence for this is that of 39 pairs of duplicate values with at least one value of 150 or more, 32 (82 per cent) had both values of 150 or more.

The lack of external regulatory review for veterinary allergy testing companies calls into question the reproducibility of the assays they provide. External peer-reviewed studies of these assays are needed, using samples from different geographical regions with IgE levels spanning the range of the assay, for example, 0 to 245 MAU for this assay, thereby providing information on the reproducibility of the assay throughout its range.

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