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Allergy testing in children with persistent asthma: Comparison of four diagnostic methods

Authors: Annica Önell PhD¹, Anna Whiteman MSc², Björn Nordlund PhD^{3,4}, Francesca Baldracchini PhD¹, Giorgio Mazzoleni PhD¹, Gunilla Hedlin MD, PhD^{3,4}, Hans Grönlund PhD², Jon R Konradsen MD, PhD^{3,4}

¹ Microtest Dx, 56 Wood Lane London, W12 7SB UK

² Department of Clinical neuroscience, Karolinska institutet, Stockholm, Sweden

³ Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden;

⁴ Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden;

Corresponding author:

Name: Hans Grönlund

Address: Department of Clinical neuroscience, Karolinska institutet, Stockholm, Sweden

Telephone: +46(0)70 4968786

Email: hans.gronlund@ki.se

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ABSTRACT

Background: Multiple allergic sensitizations are common in persistent childhood asthma, and thorough assessment of allergy is crucial for optimal care of these children. Microarray testing offers opportunities for improved sIgE characterization, which has been projected to be useful in the management of multi-sensitized patients.

Objective: The aim of this study was to investigate the accuracy and information obtained by two microarray platforms applied on a well-characterized pediatric asthma cohort.

Methods: Seventy-one (71) children were recruited from a nationwide Swedish study on severe childhood asthma. Severe (n = 40) and controlled (n = 31) asthmatics were assessed for allergic sensitization by two microarray systems (Microtest and ISAC) and by two standard diagnostic methods (ImmunoCAP and skin prick test). Data on clinical history, physical examination, spirometry, asthma control test and doctor's diagnosis were collected. Results from the four diagnostic methods were analyzed and compared.

Results: A high prevalence of allergic sensitization was observed in this cohort. The pairwise concordance between two methods was 90-92% independently of methods compared. The sensitivity of the four methods against doctor's diagnosis was 0.77-0.88, and the specificity was 0.97-0.99. Microarray methods provided new information in 47% of the sensitized children in comparison to results obtained by standard diagnostic methods.

Conclusion: The high prevalence of food and respiratory sensitization supports the clinical guideline recommendation that allergies should be evaluated in all children with suspected asthma. The microarray platforms studied here demonstrated acceptable accuracy and provided refined IgE characterization in 47% of the patients compared to standard extract-based methods.

Keywords: allergen component, microarray, multiplex, pediatric asthma, sIgE

Abbreviations:

sIgE specific Immunoglobulin E antibodies

SPT skin prick test

CAP ImmunoCAP

PR-10 pathogenesis related protein number 10

CV coefficient of variation

ISU independent standard units

INTRODUCTION

Allergies are abnormal responses by the immune system to allergenic proteins in e.g. pets, pollen or food. Symptoms of allergy can be diffuse and difficult to distinguish from other types of conditions, which in turn may lead to inaccurate diagnoses. Multiple sensitizing allergens make diagnosis even more challenging. Data suggests that more than 50% of patients consulting for respiratory allergies are multi-sensitized (1). Other data indicate that some 20-30% of the general population and >50% of asthmatic patients are allergic to common allergens (2-6). A vast number of individuals affected by allergies are children and teenagers (5,7).

Allergic sensitization is determined by measuring the concentration of specific IgE (sIgE) in blood serum or by skin prick testing (SPT) using allergen extracts to selected allergens (8-10). Allergen extracts are complex mixtures of non-allergenic and allergenic molecules (components). Extract-based tests provide information about the sensitizing allergen source, but they do not provide information on which specific allergenic component(s) present in the source, that causes the sensitization (11-14). An important advance in the understanding and diagnosis of allergy was achieved with the introduction of allergen components and microarray technologies. Allergen components may offer improved IgE characterization of patients by resolving primary allergen responses from secondary responses caused by IgE cross-reactivity (11,12,15,16). The clinical impact of microarray technologies derives from the prospects of assessing allergen sensitization to a broad panel of allergens in parallel (IgE profiling or IgE screening) using a small serum sample volume (11,16). The IgE profile of an allergen panel can provide information regarding the most relevant allergens and/or unanticipated allergens (17-19). However, a fixed panel of allergens may suffer from the absence of relevant allergen(s) and the presence of allergens that evoke unsuspected, low-level positive results without relation to clinical symptoms.

There is a need for more studies addressing the accuracy and clinical benefits of microarray-based allergy tests. The putative value of microarray testing is based on the premise of benefit from a more comprehensive and detailed sIgE profile than that obtained from 5-10 allergen extracts per patient. The main difficulties in evaluating the performance of multiplex tests are the lack of a standard

reference for comparison and the large number of allergens tested. Using allergen challenge testing to a broad panel of inhalation and food allergens as gold standard would be extremely resource demanding. The alternative strategy, which we have chosen in this study, is to compare results with results from established test methods *and* with doctor's diagnosis.

The clinical benefit of component-based allergy diagnostics using microarray technologies in children with asthma is of particular interest. Approximately 80% of children with persistent asthma are allergic, and the majority of these children are sensitized to more than one allergen (4). The presence of allergic sensitization will support the diagnosis of asthma, points to avoidable symptom triggers and targets for immunotherapy and predicts persistent and troublesome disease (6,20).

In view of the lack of data in the literature we sought to investigate the quality and accuracy of multiplex allergy tests. In this study, we compare two microarray platforms, the ImmunoCAP ISAC 112 and the Microtest Allergy System with two standard methods - ImmunoCAP and skin prick test-, and to doctor's diagnosis, in a well-characterized cohort of children with persistent asthma.

METHODS

Subjects

Children included in this study were recruited from a Nationwide Swedish study on severe childhood asthma. Children aged 6-18 (13.3 ± 2.9) years, 39 boys and 32 girls, with severe asthma ($n=40$) and age-matched peers with controlled asthma ($n=31$) were included. The two conditions were defined in accordance with guidelines established by the Global Initiative for Asthma (21). Written informed consent was obtained from all participants. The study was approved by the regional board of ethics at Karolinska Institutet (Dnr 2006/1324-31/1 and 2008/378). Details of recruitment and inclusion are presented elsewhere (4).

Data collection

Patients underwent assessment of allergic sensitization by the Microtest microarray system, ISAC microarray system, ImmunoCAP and skin prick test (SPT) in accordance with manufacturer's instructions. Sera for all measurements were drawn at the same occasion, separated within 24 h, and stored at -20°C until *in-vitro* sIgE assays were performed retrospectively. Data on clinical history,

physical examination, patient questionnaire, spirometry, asthma control test and doctor's diagnosis were collected. Test results for four food allergens (egg, milk, cod and peanut) and six inhalation allergens (cat, dog, mite, timothy pollen, birch pollen and *Alternaria*) were analyzed.

The Microtest allergy system (Microtest Diagnostics, London) is a novel microarray-based allergy platform. The system consists of a glass slide with immobilized allergens encapsulated within a fluidic chamber, a reagent cartridge, an automated instrument and software. 100 μ L of serum or plasma are required to test a predefined panel of allergen extracts ($n = 19$) and allergen components ($n = 16$) derived from 22 common food, inhalation allergens, latex and venom. The assay is automated and takes about 4 hours. A description of the method is found in (22). Serum samples were assayed from March 2014 to Aug 2015 at Karolinska Institutet, Stockholm, using a research version of the Microtest Allergy System. Responses against single allergens were analyzed in kU_A/L .

The ImmunoCAP ISAC (Phadia AB, Uppsala) system consists of a glass slide with immobilized allergens, a scanner and software. 30 μ L of serum sample are required to test a predefined allergen panel of 112 allergen components derived from 51 common allergen sources including food, inhalation allergens, latex and venoms. The assay is manual and takes about 3.5 h. A description of the technology is found in (23). Samples were assayed in 2011 at Phadia, Uppsala, using a research version of the ISAC 112 system. Results were analysed in ISU units. The ISU units correlate with the kU_A/L of ImmuoCAP units although they are not interchangeable (11,23). Both microarray methods are regulatory classified as semi-quantitative methods, however, they both provide quantitative IgE data.

The ImmunoCAP system is a well-established singleplex, allergen-specific IgE blood test (Phadia AB, Uppsala). The requesting clinician specifies how many and which allergen extracts and/or components to test. 50 μ L serum sample is required per allergen (+ dead volume for the instrument). Allergen extracts were tested with ImmuoCAP in this study, however, allergen component testing could not be performed due to lack of sample volume for many children. Results were analysed in kU_A/L .

Skin prick test (SPT) is a singleplex *in-vivo* test where the user specifies how many and which allergens to test (8, 10). Positive (histamine 10mg/ml) and negative controls were tested according to guidelines (9). The average diameter of the wheal was measured. Reagents were purchased from ALK-Abelló A/S, Denmark. Allergen components using SPT is not available. Patients taking antihistamines were excluded from the study.

Data analysis

The cut-off level for *in vitro* sIgE (blood) tests was historically set to the level of detection of the first generation of RAST sIgE tests (0.35 kU_A/L). Today the level of detection is 0.1 kU_A/L. Generally, the probability of a clinical reaction increases with increasing IgE concentration. It is documented that the clinical cut-off usually is observed at higher sIgE levels, e.g. at 1-15 kU_A/L, and varies between allergens and individuals (24-27). To decrease the number of low-level results without clinical symptoms we defined the positive cut-off for blood tests and SPT to 1.0 kU_A/L or ISU. When extract-based tests were not available, like for the ISAC test, the sum of relevant allergen components in an allergen extract was used as a substitute. This assumption has been shown to correlate well with the response from whole allergen extracts when all relevant components are included (22,23).

IgE results generated by the four methods were compared by analyzing

- a) Positive/negative concordance; calculated as the sum of positive and negative observations in agreement between two methods expressed in percentage
- b) Quantitative agreement (SPT excluded) including Spearman rank correlation coefficients (r) and p-values
- c) Discrepant results

Discrepant results were compared to the reference status defined by at least three diagnostic methods giving the same indication (*Majority IgE status*) and then compared to the status defined by doctor's diagnosis (*Dr's Diagnosis*). A positive/negative *Majority IgE status* was defined by three to four methods giving the same positive/negative result. Inconclusive results were defined either as opposing results from methods (two methods giving positive results, and the other two returning negative results, or as one discrepant result among three results in case of a missing value).

The diagnoses of asthma were made by the referring Pediatric Allergists. The children referred were then invited to participate in this study, and, following reassessment of their medical histories, medication and adherence to prescribed treatment, final selection of the participants was performed according to the criteria. Doctor's "allergen-specific" diagnosis was based on clinical history, physical examination, patient questionnaire data and optional SPT and/or *in vitro* test (ImmunoCAP) used in the ordinary routine at the clinic. A positive diagnosis required IgE antibodies towards the specific allergen and a clinical history of allergic symptoms upon exposure. Final Drs Diagnosis for each of the 10 allergens x 71 patients studied was reviewed and approved by the allergists in the research

study team. The microarray test results were unknown at the time of the diagnosis. A reformulation (since a substitute variable was used as a gold standard) of the sensitivity, specificity, positive/negative predictive value (PPV/NPV), and the positive and negative likelihood ratios were calculated as described in (27)). Samples with status “Inconclusive” Dr’s diagnosis were excluded from the calculations.

RESULTS

Ten common allergens were tested with SPT and ImmunoCAP. Consequently, the comparative analysis between the methods (SPT, ImmunoCAP, ISAC and Microtest) was restricted to these 10 allergens despite both microarray systems generating results for more allergens. The prevalence of the 10 allergens studied is shown in Figure 1. The maximum number of observations (n) per method was 710 (71 patients x 10 allergens). There were a few missing observations due to individual allergens not tested by ImmunoCAP and SPT (mainly *Alternaria*) and one excluded sample for ISAC (n(Microtest) = 710, n(ISAC) = 700, n(ImmunoCAP) = 661 and n(SPT) = 668).

Patient characteristics

Among the 71 children, 53 (75%) children had an allergic asthma. 19 (27%) children were sensitized and allergic to one or two of the ten allergens. The remaining 34 (48%) children were multi-sensitized and diagnosed allergic to 3-8 allergens. Nearly one in five (~18%) of the patients were sensitized to five or more allergens.

Figure 1. Prevalence of allergy/IgE sensitization according to doctor’s diagnosis, Microtest, ISAC ImmunoCAP and SPT.

Concordance between methods

A total of 3964 pairs of observations were analysed and the positive/negative concordance observed was 90–92% independent of which two methods compared. The quantitative IgE agreement between methods was further analyzed. Results obtained for ImmunoCAP allergen extracts were compared to their counterpart measured by ISAC and Microtest (Figure 2). (SPT results were excluded from this exercise due to different measurement units.) The quantitative agreement between allergen components as measured with the two microarray methods is shown in Figure 3. Figure 2 and 3 includes the Spearman rank correlation coefficient (r) with statistically significant p-values ($p < 0.0001$).

Figure 2. Allergen sources: Quantitative comparison with ImmunoCAP IgE concentrations on the x-axis versus ISAC and Microtest sIgE concentrations on the y-axis for different allergen sources and the Spearman correlation coefficient (r). Each patient's results are represented by two dots; filled diamonds represents ImmunoCAP versus Microtest and unfilled squares represents ImmunoCAP versus ISAC.

Figure 3. Allergen components: Quantitative comparison with Microtest IgE concentrations on the x-axis versus ISAC IgE concentrations on the y-axis and the calculated Spearman correlation coefficient (r) for different allergen components.

Accuracy of methods compared to majority IgE status

The accuracy of the methods ability to detect sIgE sensitization to a species, i.e. to the whole allergen extract, was assessed. A positive/negative agreement where all four methods gave the same indication was found for 593 of 710 observations (84%). Out of 710 observations the number of discrepant *positive* results ranged from 10 (ISAC), 11(SPT), 13(Microtest) to 26 (ImmunoCAP). The number of discrepant *negative* results compared to *Majority IgE status* was 3 for all *in-vitro* blood tests and 23 for SPT, whereof 18 of the 23 showed high sIgE responses reported by all three *in vitro* test methods ($> 15 \text{ kU}_A/\text{L}$). This outcome is examined further in the Discussion.

Accuracy of methods compared to Dr's Diagnosis

The accuracy of the test in predicting clinical reactivity was investigated. Out of 710 observations the number of discrepant *positive* results compared to Dr's diagnosis ranged from 6 (SPT), 11(ISAC), 14(Microtest) to 15 (ImmunoCAP). See Supplementary Table 2. The number of discrepant *negative* results ranged from 21(ImmunoCAP), 28(ISAC), 30(Microtest) to 41 (SPT).

The reformulated positive/negative predictive value (PPV/NPV)), sensitivity and specificity values, and positive/negative likelihood ratios (LR) at cut-off $1.0 \text{ kU}_A/\text{L}$ for the different test methods are presented in Table 1.

New IgE information provided by microarray results

New information was obtained in 26 out of 55 (47%) sensitized children when allergen component information as well as testing a panel of allergens was taken into account. In eighteen patients IgE antibodies to Ara h 2, a storage protein in peanut, were identified. Six patients had IgE antibodies to Ara h 8, a cross-reactive birch pollen related PR-10 protein. Multiple sensitization to cat, dog and horse derived lipocalins was diagnosed in 8 children, and 7 patients revealed sensitization towards less common allergens such as mold or mite. In 9 multi-sensitized patients with 5-8 sensitizing food and respiratory allergens the microarray-based tests made it possible to compare the sIgE levels to several species-specific, pan and cross-sensitizing allergens simultaneously.

DISCUSSION

This study explored allergic sensitization in a group of children with persistent asthma using four different diagnostic methods: Microtest, ISAC, ImmunoCAP and SPT. The uniqueness of the study lies in the completeness of the dataset with very few missing measurement values. The major findings are a) a high prevalence of allergic sensitization in this asthma cohort, b) a 90-92% concordance of test results between pair-wise diagnostic methods, c) no significant differences in accuracy was observed for the two microarray methods when compared to standard allergy diagnostic tests (ImmunoCAP and SPT), and d) new sIgE information was found in 47% of the sensitized patients when using the allergen component information provided by the microarray tests.

A high prevalence of allergic sensitization in the study group was observed. 53 of the 71 (75%) children were diagnosed allergic, and 34 of the 53 allergic children (64%) revealed multiple sensitizations to 3 to 8 allergenic species, whereof 22 children demonstrated at least one food allergen. The high prevalence of food and respiratory sensitization supports the clinical guidelines recommendation that allergies should be evaluated in all children with suspected asthma (20,28).

Evaluating the accuracy of IgE microarray systems is not trivial. In this study we found pair-wise concordances of 90-92% between methods. This is in line with previous studies, which generally show 80-90% concordance between methods (22,25,29-35). All allergens and all methods revealed discrepant results at low levels (<2 kU/l and <4 mm), most likely due to differences in allergen preparation and assay/measurement technologies. Egg, milk, mite, cod and mold show a low prevalence with less than 9 positive samples each while a high prevalence was noted for cat, birch,

timothy, dog and peanut (Fig.1). Unexpectedly low prevalences were noted for birch on SPT (further discussed below) and dog on ISAC (Table 2). For birch SPT: 18 clinically relevant and highly positive allergens as defined by doctor's diagnosis and all three in-vitro methods (15-100 kU_A/L) were identified. The company producing the SPT extract confirmed that at the time and in the region of the study, there were problems with the quality of birch extracts. (Ref: letter from ALK). These data are based on one SPT provider during a limit period of time, however, it underscore the critical importance of the quality and standardization of allergens. For dog on ISAC: Out of 28 samples with positive Drs diagnosis and IgE levels between 0,87 kU/l - 8.7 kU/l on ImmunoCAP 13 were negative on ISAC. The low prevalence of dog on ISAC may be explained by some dog components not present on the microarray. Further, the IgE concentration measured with Microtest was higher than ImmunoCAP and ISAC for Phl p 5, Bet v 1, Ara h 2, dog, birch and timothy (Fig.2 and 3). The calibration of these allergens may need further optimization by the manufacturer.

Challenge confirmed data, for the food and inhalation allergens studied, should optimally have been collected to define the true status of triggering allergens. Challenge data, collected at the same time period as SPT was performed and blood where drawn, do not exist. This is a drawback of the study, however, the experimental risk to induce severe reactions in children with severe asthma was not warranted by the benefit. Due to lack of challenge data we decided to use "Doctor's diagnosis" and "Majority IgE status" as substitute variables and to analyze different measures of accuracy with different advantages/disadvantages to draw conclusions. At a cut-off level 1 kU_A/L for in-vitro blood tests and 3 mm wheal diameter for SPT, the observed differences between methods were statically non-significant. We conclude that the four methods show comparable accuracy to detect IgE to 10 common allergen sources/extracts at these cut-offs. Lower cut-off levels, e.g 0.35 kU_A/L or 0.1 kU/l, are associated with increased sensitivity and numerous positive results without clinical symptoms (19,24,26,27). It should be noted that the results of SPT and ImmunoCAP are slightly biased as they were used as an aid when defining doctor's diagnosis, however, we chose to include them in Table 1 for the purpose of comparison. It should also be noted that Table 1 includes uncertainties/errors since substitute variable for the true clinical status were used.

New information was provided by the microarray results compared to traditional extract-based tests. Detection of IgE antibodies towards Ara h 2 and Ara h 8 could be of clinical relevance as sensitization against Ara h 2 is associated with systemic reactions upon exposure to peanuts (12,15,36,37), whereas sensitization to Ara h 8 is associated OAS (12,38). Unexpectedly, the majority of peanut sensitized patients demonstrated sensitization to Ara h 2 and not to Ara h 8 (which is common in our geographical region). The 8 patients with multi-sensitization towards lipocalins are more likely to

have more severe allergic disease (39-41). Further, allergy to molds and mite is uncommon in our region and can be difficult to identify based on clinical history alone. The diagnosis of sensitization to these allergens can hold valuable implications for exposure control and avoidance measures. In the multi-sensitized individuals, the ability of the microarray to assay sIgE towards a panel of species-specific, and broadly cross reacting allergens from food and inhaled allergens, allows the clinician to distinguish between those allergens that are markers of cross-reactivity with minor clinical importance and those allergens that most likely to cause symptoms. The latter could be potential targets for allergen avoidance and immunotherapy. Finally, for those patients who's clinical history suggests allergy, but who are found not to have IgE antibodies against a few common allergens assessed by SPT or ImmunoCAP, a more extensive assessment via a broad panel of allergens may help the clinician to rule out allergic sensitization, or to identify sensitization to uncommon and unsuspected allergens which the clinician normally would not consider to test for. Similar information can be obtained by ImmunoCAP single testing if all relevant allergens are requested.

The number of patients with new, potentially relevant IgE information will vary depending on the cohort studied. We found five studies in the literature that have investigated the value of microarray testing. These studies are in line with the results found here. One study (n=100, UK) reported that microarray testing helped to identify the unknown, culprit allergen in 20% of patients with idiopathic anaphylaxis (18). Two studies (n=55, Spain (42); n=318, Italy (43)) reported that microarray testing revealed new IgE information leading to a more confident diagnosis in 91% and 93% of patients respectively. Three studies assessed the specific immunotherapy (SIT) prescription decision based on SPT versus microarray testing and reported a difference of SIT prescription decision in 28% (Italy)(43), 54% (Spain)(44), and 54% (Spain)(45) of patients, however, none of the three studies have followed-up and reported the clinical outcome *after* SIT treatment.

The high prevalence of food and respiratory sensitization supports the clinical guidelines recommendation that allergies should be evaluated in all children with suspected asthma. Economic considerations and limitations, number of allergens to be tested, amount and type of information gained and sample volume required are factors that may influence the decision whether to use a singleplex or multiplex investigation. (The cost of a microarray-based test is usually similar to the cost of 10-12 single ImmunoCAP tests.) For non-sensitized or mono-sensitized patients with a relatively clear clinical history singleplex methods will likely suffice and be a cost efficient approach. If >10 allergens needs to be tested to rule out allergy then multiplex tests may offer an efficient alternative. For multi-sensitized patients with IgE to both food and inhalation allergens, multiplex testing including components may offer a cost efficient alternative to obtain a high resolution "IgE

image” of the patient. The disadvantage of testing broad allergen panels is the likelihood of revealing unsuspected sensitivities, possibly to potentially harmful allergens. Although the sensitization may be relevant the interpretation of such results may be difficult. As the number of allergens measured increase, or the limit of detection decrease, the number of detectable, asymptomatic sensitizations will increase. Appropriate training is therefore required to interpret the results in conjunction with the clinical information of the patient.

Author contributions

Annica Önell is responsible for the study design, data evaluation and manuscript writing. Anna Whiteman ran and evaluated the Microtest assays, and contributed with literature and manuscript writing. Björn Nordlund contributed to the SPT, ImmunoCAP and ISAC data collection. Francesca Baldracchini and Giorgio Mazzoleni contributed with microarray data analysis and figures. Gunilla Hedlin contributed to the overall scientific concept and patient cohort collection. Hans Grönlund contributed to the study design, data collection and scientific concept. Jon Konradsen is responsible for the patient data collection and characterization and contributed to the scientific concept, result visualization and manuscript writing.

Conflicts of interest

Björn Nordlund and Anna Whiteman declare no conflict of interests. Gunilla Hedlin and Jon Konradsen has participated in symposia and been invited speakers at conferences/meetings organized by Thermo-Fisher Scientific and received honorarium. Hans Grönlund was previously appointed to Microtest Scientific Advisory Board with a consultant contract. Annica Önell, Francesca Baldracchini, Giorgio Mazzoleni are employed at Microtest Diagnostics (London). Annica Önell was employed at Phadia AB/ThermoFisher Scientific (Uppsala, Sweden) in 2007-2014.

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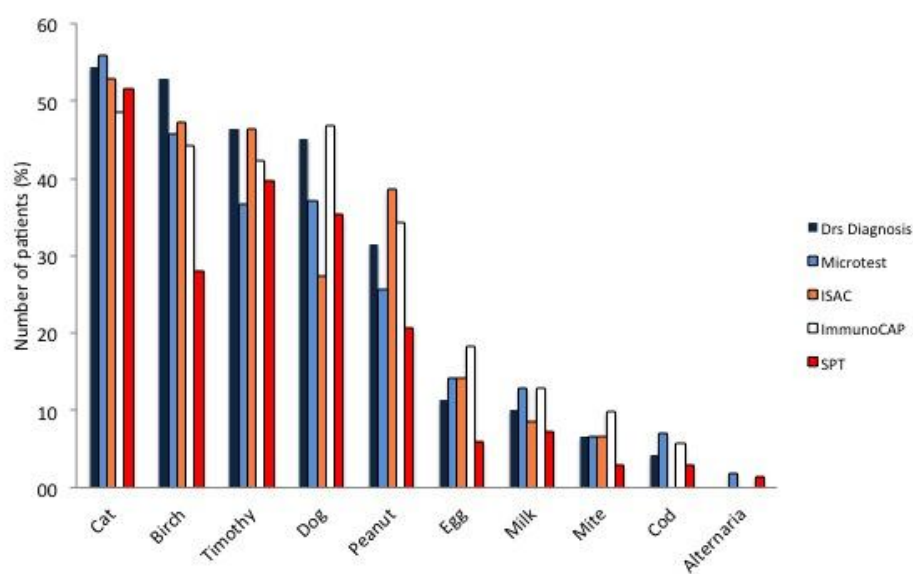
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Table 1. Calculated sensitivity, specificity, PPV, NPV and likelihood ratios

	Sensitivity	Specificity	PPV	NPV	LR+	LR-
Based on Majority IgE status						
Microtest	0.98	0.97	0.92	0.99	32.67	0.02
ImmunoCAP	0.98	0.95	0.85	0.99	19.60	0.02
ISAC	0.98	0.98	0.94	0.99	49.00	0.02
SPT	0.84	0.98	0.92	0.96	42.00	0.16
Based on Dr's Diagnosis						
Microtest	0.83	0.97	0.91	0.94	27.67	0.18
ImmunoCAP	0.88	0.97	0.91	0.96	29.33	0.12
ISAC	0.84	0.98	0.92	0.94	42.00	0.16
SPT	0.77	0.99	0.97	0.92	77.00	0.23

Figure 1



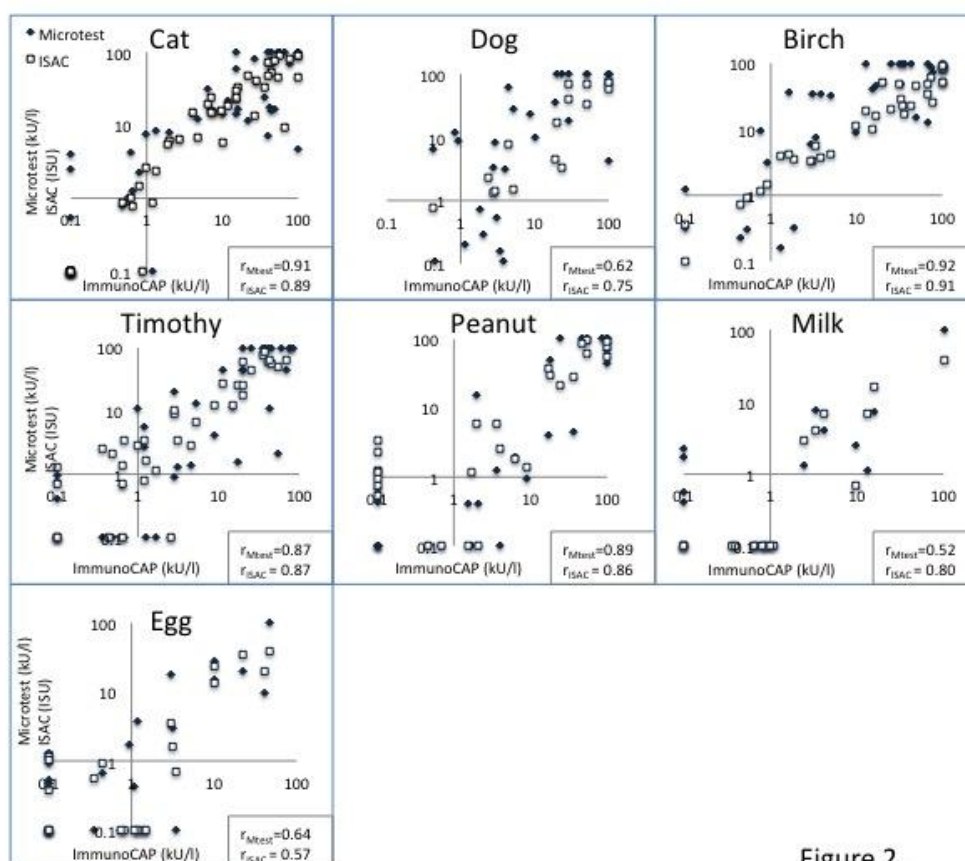


Figure 2.

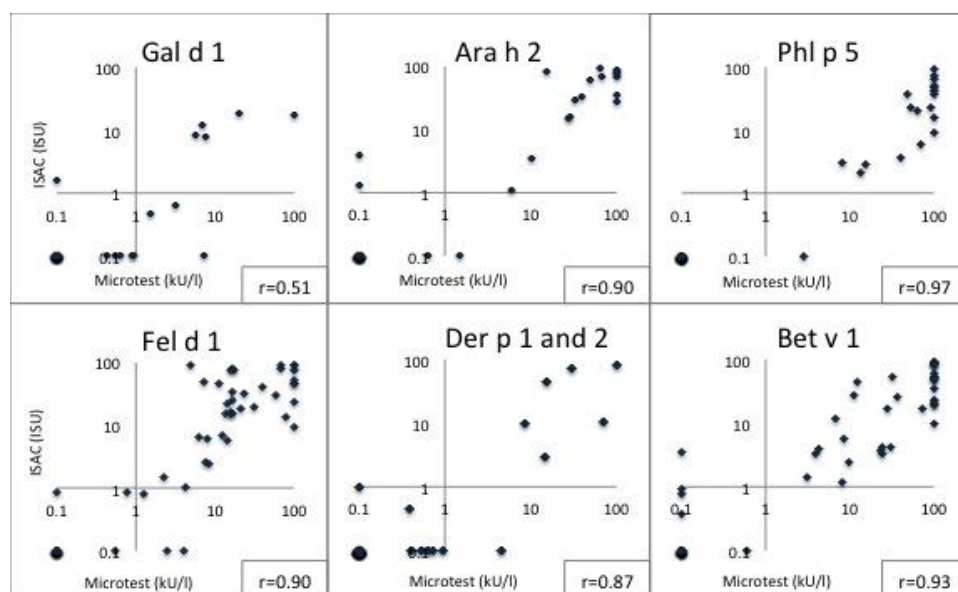


Figure 3.