Qualitative and quantitative comparison of allergen component-specific to birch and grass analyzed by ImmunoCAP assay and Euroline immunoblot test

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ABSTRACT

Background: In the diagnostic work up of allergy, determining allergen component-specific immunoglobulin E (IgE) is important for diagnosis, prognosis and choice of treatment.

Objective:

The purpose of this study was to evaluate the performance of the immunoblotting assay (Euroline) in detection of IgE antibodies against timothy grass and birch pollen allergen components compared to fluorescent enzyme assay (ImmunoCAP, Phadia 250).

Methods: A total of 128 serum samples from patients allergic to timothy grass and birch pollen were analysed. The levels of IgE antibodies to timothy grass and birch pollen were measured using Euroline DPA-Dx pollen 1 and ImmunoCAP assay. The two methods were then compared on binary (positive vs. negative), semi-quantitative (IgE classes) and quantitative (concentration) levels. The two methods were also compared to results from skin prick testing.

Results: The Euroline method showed a positive percentage agreement of 93% and negative percentage agreement of 94% with an overall accuracy of 94% when compared to ImmunoCAP. Kappa analysis showed moderate strength of agreement between the methods in determining IgE classes for 7/11 components tested. All components showed a positive correlation when analysed using Spearman's rank correlation.

Conclusion: Overall, we found that there is good correlation between the Euroline and ImmunoCAP methods in measuring IgE sensitization.

Keywords: Pollen, Allergen specific IgE, ImmunoCAP, Euroline

Impact Statement: Use of the Euroline method may be recommended as an alternative routine clinical allergy diagnostic work up to determine sensitization profiles to timothy grass and birch pollen.

INTRODUCTION

It is challenging to diagnose allergic diseases especially when there are contradictory outcomes between clinical and laboratory findings. Thus, correct diagnosis requires good agreement between the clinical features and serological tests. Accurately detected specific Immunoglobulin E (IgE) sensitization and specific IgE profiling, is not only essential to diagnose allergic patients but it also has a key impact on optimal decision making for successful allergen immunotherapy strategies tailored for the individual patient [1]. The use of allergen components is of great diagnostic importance for identifying the major sensitizing component, especially when the results of allergen-specific IgE mismatch with the subjective allergic symptoms of the patient. As a result, the risk of serological cross-reaction and/or over-interpretation of the results is reduced.

Various methods exist for testing allergen-specific IgE antibodies and their results can vary greatly, thereby affecting both the diagnosis and treatment of allergic diseases [2]. It is not easy to compare the results of different test systems with each other as there are differences in the development of the methods. An ideal assay method would be simple and easily carried out, time- and cost effective, and most importantly, of the highest performance.

The singleplex immunoassay ImmunoCAP (Thermo Fischer) specific IgE measuring assay system is used worldwide as a diagnostic test for allergy. It is the most widely evaluated method and its sensitivity, specificity, and positive predictive values have been shown to be over 90%, while other methods are less focused [1]. However, the multiparameter assays for specific IgE assay (Euroline), are used with increasing frequency [3,4]. This test system offers the advantage that allergen-specific IgE antibodies, against multiple pollen allergens, can be determined semi-quantitatively in a single serum incubation.

The aim of this study is to describe the effectiveness of Euroline compared to ImmunoCAP in identifying levels of allergen-specific IgE antibodies to timothy grass and birch pollen, and to evaluate Euroline as a potential alternative method for routine clinical testing of allergen-specific IgE. The two methods will be compared in relation to the subjects' allergic profiles, as well as how the subjects have responded to allergen immunotherapy (AIT).

MATERIALS AND METHODS

Study design and population

This study is a retrospective study based on a cohort of 128 adult patients with medical history of allergic rhinitis, positive skin prick tested (SPT) and/or allergen-specific IgE test for timothy grass and/or birch pollen allergy who were set to undergo AIT targeting grass and/or birch allergy. This cohort has been used previously by our group [4]. This study has received approval by the Swedish Ethical Review Authority.

Skin prick test

SPTs were carried out using a panel of commercially available extracts (Soluprick SQ®, ALK – Abelló; Hørsholm, Denmark).

Detection of the allergen components

Serum from the 128 subjects was analysed using two different methodologies (ImmunoCAP and Euroline). As the testing occurred prior to designing this study, there is some mismatch between the components tested with each method. Only subjects where there was enough serum sample to perform analysis with both methods were included in the analysis of each specific allergen component. A total of 1364 paired tests were performed on the 128 serum specimens.

Method 1 (ImmunoCAP, Phadia 250)

Serum samples stored at −20 °C were analysed for allergen-specific IgE antibodies with ImmunoCAP Fluoro Enzyme Immuno Assay (FEIA) (Thermo Fisher Scientific/Phadia AB, Uppsala, Sweden) according to the manufacturer's recommendations. The samples were analysed for specific IgE against *Phleum pratense*, timothy (g6) and its allergenic components rPhl p 1, rPhl p 4, rPhl p 5b, rPhl p 6, rPhl p 7 + rPhl p 12; against *Betula verrucosa*, birch (t3) and its allergenic components rBet v 1, rBet v 6, rBet v 2 + rBet v 4 and MUXF3 CCD, Bromelin. These components for birch and timothy pollen allergy were chosen according to ThermoFisher's recommendation. The result for each allergen is stated in kU/L, the limit of detection is 0.1 kU/L and is divided into the following classes: 0 (< 0.35 kU/L), 1 (0.35 kU/L to < 0.7 kU/L), 2 (0.7 kU/L to < 3.5 kU/L), 3 (3.5 kU/L to < 17.5 kU/L), 4 (17.5 kU/L to < 50 kU/L), 5 (50 kU/L to < 100 kU/L) and 6 (> = 100 kU/L). Controls for timothy and birch with

known concentrations have been run at each analysis to ensure that the method works as intended.

Method 2 (EUROBlotOne, Euroline DPA-Dx pollen 1)

Serum-specific IgE antibodies were measured with EUROBlotOne, EUROLINE DPA-Dx pollen 1 (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany) according to the manufacturer's instructions, and the results expressed in kU/L with a lower limit of detection of 0.35 kU/L and an upper limit of 100 kU/L. The results were divided into classes in the same way as method 1. The test kit contained strips marked with parallel lines with 11 different allergens and a control line (indicator band). Serum samples were analysed for specific IgE against *Betula verrucosa*, birch (t3) and the birch components rBet v 1, rBet v 2, rBet v 4, rBet v 6; *Phleum pratense*, timothy (g6) and the timothy components rPhl p 1, rPhl p 5, rPhl p 7, rPhl p 12 and the cross-reactive carbohydrate determinants (CCDs). A known control sample, positive for birch (t3) and timothy (g6) was run with each analysis to ensure that the method worked as intended.

Statistics

Microsoft® Excel® was used to store the data and to create the tables presented in this study. All interval or ordinal level variables are presented as median (min–max). Categorical level variables are presented as frequencies (percentage).

All statistical analysis was conducted in R, a software environment for statistical computing [5]. To compare the two methods' agreements in determining the semi-quantitative classes of specific IgE concentration, Cohen's kappa values were calculated [6]. To compare the performance between the two methods of measuring the concentration of specific IgE, a Spearman correlation analysis was performed [7]. To visualise the quantitative agreement between the two methods, both conventional scatter plots, as well as Bland-Altman plots were constructed [8]. As a crude measure of clinical significance, sIgE levels were compared to AIT outcome using Fisher's exact test, as previously done by the authors for the Euroline test method [4].

As the lower and upper limits of detection are different for the two methods, IgE concentration value < 0.35 kU/L was converted to 0.34 kU/L and value $\geq$ 100 kU/L was converted to 100 kU/L prior to statistical testing.

Results

Out of the 128 study subjects, 77 (60%) were female. The median age of the patients was 33 years, ranging from 17 to 70 years. A total of 61 (48%) subjects reported suffering from asthma, while 34 (27%) patients had an asthma diagnosis according to the medical records. Positive skin prick tests (SPT) for timothy grass were seen in 114 patients, and for birch in 105 patients. In three patients, information about SPT results could not be found in the medical records. One patient had tested negative with SPT for both timothy grass and birch. The vast majority of the patients were polysensitized (n=117) with a positive SPT for two or more allergens (grass and birch, or grass or birch plus some other allergen). Baseline characteristics of the study subjects are shown in Table I.

The overall accuracy of Euroline compared to ImmunoCAP was 94%, with a positive percentage agreement (PPA) of 93% and negative percentage agreement (NPA) of 95%. The lowest PPA was seen when comparing Euroline Phl p 7 to ImmunoCAP Phl p 7+ Phl p 12 (20%) and Euroline Bet v 4 to ImmunoCAP Bet v 4 + Bet v 2 (17%). The performance of Euroline assays compared to ImmunoCAP on a nominal level (positive vs. negative), can be seen in Table IIa. Similar high performance was observed when positive IgE to g6 and t3 was compared to grass and birch sensitization according to SPT (Table III).

For cross-reactive carbohydrate determinants (CCDs), the number of positive subjects was generally low for both test methods. 7 subjects were positive for CCDs according to both Euroline and ImmunoCap, while 10 was only positive according Euroline and 1 only according to ImmunoCAP (positive predictive value, PPV 41%). However, the overall accuracy of Euroline in determining positivity for CCDs was high (91%). For the 11 subjects with discordant results on the CCD analysis, no obvious differences were seen for the other components (table IIb).

There were 9 subjects who were positive for Bet v1 according to Euroline while negative according ImmunoCap. Out of these 9 subjects, 5 had shown positive SPT to birch.

To assess the inter-rater agreement between Euroline and ImmunoCAP assays in determining the semi-quantitative classes of IgE concentration, weighted Cohen's kappa coefficients (κ_w value) were calculated (Figure 1). The lowest κ_w value was observed for the comparison of Euroline v4 to ImmunoCAP v 4 + v 2 (0.27; 0.20–0.34) and the highest for Euroline Bet v 6 to ImmunoCAP Bet v 6 (0.74; 0.66–0.83).

Spearman rho coefficients (r_s) between the two methods were calculated for each component (Figure 2). This analysis showed positive r_s values with p-values less than 0.05 for all components tested. The lowest r_s values were found when comparing Euroline Phl p 7 to ImmunoCAP Phl p 12 + Phl p 7 ($r_s = 0.44$, $p < 0.001$) and Euroline Bet v 4 to ImmunoCAP Bet v 4 + Bet v 2 ($r_s = 0.39$, $p < 0.001$). The highest r_s value was found when comparing Euroline Phl p5 to ImmunoCAP Phl p5 and Euroline Bet v1 to ImmunoCAP Bet v1 ($r_s = 0.96$, $p < 0.001$). As shown in figure 2, Euroline showed a positive bias compared to ImmunoCap for most of the tested components.

There were no statistically significant differences between AIT outcomes and IgE-levels as measured by ImmunoCAP (Table IV).

Discussion

Proper diagnosis of clinical allergy and effective treatment are critical for patients with allergy [9]. This requires correct diagnosis of an IgE-mediated disease and a clear connection between the identified allergen and the patient's symptoms.

The singleplex assay ImmunoCAP system has high analytical sensitivity (lower limit of quantitation) and greater sensitivity at low specific IgE levels. It needs only 40 µL serum or plasma per individual test [1]. However, it can be criticized for being expensive, requiring individualized testing and having a lengthy testing time [10]. As an alternative, a single test for multiple allergens using customized allergen profiles (Euroline) has been introduced in clinical practice as a reliable and cost-efficient specific IgE test with acceptable correlation with ImmunoCAP. It is simpler and faster than the ImmunoCAP system, and requires a small serum volume (100–200 µL) to provide results for multiple allergen components and to get a screening overview of the patient's sensitization [3,11,12]. The method is gaining increasing clinical awareness [3,4]. However, when looking at scientific publications in the Medline database published before 2020, ImmunoCAP heavily outweighs Euroline with 600 publications compared to 7 articles about Euroline [2].

Because of the underlying different methodological backgrounds, it is not surprising that differences appear between different immunoassays in terms of sensitivity and specificity or in terms of IgE concentration. These may be due to differences in method sensitivity, the use of native or recombinant allergens and the representation of the sensitizing molecule in the testing procedure [1,13]. This may cause some confusion when it comes to the interpretation of the test results. Hence, we evaluated the allergen-specific IgE antibody-

detection performance of the Euroline immunoblot test in comparison to ImmunoCAP system.

Our study found that on a binary level (i.e., positive vs. negative) the Euroline test method has good concordance with the ImmunoCAP method. The PPA of Euroline compared to ImmunoCAP was > 80% for 8/11 tested molecules, and the NPA > 80% in 9/11 molecules. The cumulative PPA and NPA for all molecules were 93% and 95%, respectively. The cumulative accuracy in comparison to ImmunoCAP was 94%. These findings indicate that the Euroline method is reliable in testing specific IgE sensitization and is in accordance with previous studies [1,3].

To make the comparison more impartial and interesting, we tested how these two methods performed when compared to results from SPT. When comparing SPT positivity for grass to IgE positivity for g6 and SPT positivity for birch to IgE positivity for t3, both the Euroline and ImmunoCAP methods showed similar accuracy (Table III). This outcome outlines that the Euroline method is a reliable alternative method to the gold standard method ImmunoCAP in testing for IgE sensitization.

When comparing the IgE classes as determined by the two methods, the κ_w value was > 0.40 for 7/11 subjects. The highest κ_w value was found for v6 (0.74). According to a traditional interpretation of kappa coefficients a value > 40 would be considered a moderate strength of agreement, while 0.74 would be considered a substantial strength of agreement. However, some researchers deem this interpretation to attribute too much strength to low coefficients. According to McHugh [14] a kappa coefficient > 40 should instead be considered a weak level of agreement and > 60 as a moderate level of agreement.

When comparing IgE concentrations as determined by the two methods, all tested allergen components showed a positive r_s value with a p-value < 0.05, indicating that there was indeed a positive correlation. Further, 6/11 tested allergen components showed r_s values > 0.8, which can be described as a very strong relationship [15]. Only two of the tested components showed r_s values between 0.3 and 0.5, which can be considered a fair strength of relationship. Furthermore, these two r_s values were found when comparing a single component tested with Euroline (Phl p 7 and Bet v 4) with multiple components tested with ImmunoCAP (Phl p 12 + Phl p 7 and Bet v 4 + Bet v 2). These findings can potentially be related to the fact that two different sets of components were compared, as a single component in Euroline was compared to a combination of two components in ImmunoCAP.

These outcomes could have been quite different if ImmunoCAP's single component analysis for Phl p 12, Phl p 7, Bet v 4, and Bet v 2 components had been used instead.

No clear association was found between AIT outcome and pre-treatment IgE levels as measured by ImmunoCAP (Table IV). This is similar to the results previously published by the authors concerning association between AIT outcome and IgE levels as measured by Euroline [4].

An advantage with the Euroline immunoblot test is that the strip includes the cross-reactive carbohydrate determinants (CCD) marker; with ImmunoCAP, CCD specific IgE must be tested separately. CCD is present in many allergens with reactivity in 7.5–35% of the patients. It has little clinical relevance, but can be a problem in diagnosis, as the presence of IgE towards CCDs may cause a false-positive reaction due to interference or cross-reactivity in allergen-specific IgE assays [2]. Therefore, the CCD marker may provide useful information, especially with positive-specific IgE results that disagree with the clinical picture, and can aid in interpreting overall test results showing concurrent positivity for multiple allergens [12,16].

Although most samples (91%) show qualitatively the same anti-CCD results according to both Euroline and ImmunoCap methods, there were eleven samples (9%) with discordant results on a negative to positive (>0,35 kU/L)-scale (table IIa). In one way it is preferable not to find too many positive CCD-reactions since a true CCD-result may cast some shadow on the clinical importance on the results for naturally derived allergens in the same sample, while that is not a problem for recombinant allergen components. On the other hand, it is preferable to receive a CCD-reaction, if it is a true one, if the presence of anti-CCD antibodies may be a reason for false positive reactions in analyses for single allergens in the same sample. False positive reactions to single allergens may result in a false clinical interpretation and unnecessary avoidance of harmless allergens.

There are different approaches how to handle a positive CCD result in clinical routine situations. One way is to make further analyses with recombinant components, another way is an absorption of anti-CCD antibodies before analysis of IgE against the single allergens. In some cases, a "second opinion" by another method may be valuable. However, if it is a true anti-CCD reaction and both sources of naturally derived allergens contain anti-CCD antibodies this way may not be a valuable option. In most clinical situations the results

from analysis of IgE to allergens are considered together with the clinical context, and if valuable and possible a common choice is analyses with recombinant allergen components.

For the 11 subjects with discordant results to CCD, there were no obvious increase in discordant results for the other analysed components (table IIb). This was to be expected as all single components analysed in this study were recombinant allergen components.

Visual inspection of Figures 1 and 2 shows that on both a semi-quantitative and quantitative level (i.e., measuring IgE classes and IgE concentration, respectively) the Euroline method tended to show higher values compared to the ImmunoCAP method. This is shown clearly in the Bland-Altman plots presented in figure 2, where Euroline shows a positive bias compared to ImmunoCap for most of the tested components. This finding might be due to manufacturing and calibration differences between the two methods [3]. A difficulty in comparing these two methods is that the two methods have different limits of detection. Euroline only measures IgE concentrations between 0.35 kU/L and 100 kU/L, while ImmunoCAP measures IgE concentrations > 0.1 kU/L. In clinical practice, concentration values below 0.35 kU/L are usually considered as negative, class 0; however, this is not an absolute limit but rather expresses a probability of allergy. Thus, it could be considered of importance to be able to measure concentrations less than 0.35 kU/L.

Overall, caution should be exercised when comparing results measured with different methods. Thus, despite the relatively good correlation between these two methods that has been shown in this study, there may be other discrepancies between these test systems which could not be ruled out.

Conclusion

According to the findings of the present study, we conclude that the Euroline method performs well and is concordant with ImmunoCAP in determining sensitization to birch and grass pollen allergen molecules. Furthermore, Euroline shows acceptable correlation to ImmunoCAP in determining specific IgE concentration for birch and grass pollen molecules. Use of the Euroline method may be recommended as an alternative routine clinical allergy diagnostic work up to determine sensitization profiles to timothy grass and birch pollen.

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Disclosure statement

No conflict of interest exists.

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Figure 1. Inter-rater agreement between Euroline and ImmunoCAP methods for determining IgE classes (0–6) to different allergen specific IgE. Numbers in **bold** represents IgE classes and numbers in central grid represent frequencies.

κ_w – weighted Cohen's kappa coefficient; CI – confidence interval

Figure 2. Correlation between concentration of allergen-specific IgE, as measured by either Euroline or ImmunoCAP method. Presented with scatter plots visualising the concentration according to the different methods on the left, and Bland Altman plots to the right.

r_s – Spearman's rho coefficient