Interleukin-4 enzyme-linked immunospot assay may be useful for diagnosing sensitization to house dust mite

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Background: **The skin prick test (SPT) is considered a standard test for identification of allergens, but it has some limitations in clinical practice. The multiple allergen simultaneous test (MAST), which measures allergen-specific immunoglobulin E in patients' serum, is a widely used alternative test, but is limited by its relatively low sensitivity and specificity. As a novel diagnostic test to identify allergens, we investigated the sensitivity and specificity of an interleukin-4 (IL-4) enzyme-linked immunospot (ELISpot) assay for *Dermatophagoides farinae* (*Der f*) and *Dermatophagoides pteronyssinus* (*Der p*).**

Methods: **Based on the symptoms and SPT results, 43 house dust mite (HDM) allergic rhinitis (AR) patients and 41 control subjects were included. Peripheral blood was drawn from each subject for IL-4 ELISpot assay and MAST. The receiver operating characteristic (ROC) curve analysis was conducted to determine the cutoff values. Sensitivity, specificity, and positive and neg predictive values were compared between the 2 tests.**

Results: **The sensitivity, specificity, and areas under the ROC curve (AUCs) of the IL-4 ELISpot assay were 88.4%, 97.6%, and 0.939 for *Der f*, and 95.3%, 97.5%, and 0.971 for *Der p*, respectively. However, the sensitivity, specificity, and AUC of MAST were 76.7%, 73.2%, and 0.777 for *Der f*, and 69.8%, 75.6%, and 0.788 for *Der p*, respectively.**

Conclusion: **The IL-4 ELISpot assay showed higher sensitivity, specificity, and AUC than MAST, which indicates its clinical feasibility for diagnosing allergy for HDM. A further study is needed to determine the accuracy of the IL-4 ELISpot assay for other common allergens.** © 2016 ARS-AAOA, LLC.

Key Words:
allergy; allergic rhinitis; enzyme-linked immunospot assay; house dust mites; interleukin-4

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Allergic inflammation occurs when the immune system responds to normally harmless substances called allergens. It is initiated by activation of specific white blood cells, including mast cells and basophils, in the presence of allergen-specific immunoglobulin E (IgE).[1] Because allergen-specific IgE is needed to elicit allergic inflammation, several in vivo or in vitro tests have been developed to determine its presence; these include the skin-prick test (SPT), multiple allergen simultaneous test (MAST), and radioallergosorbent test (RAST), which is currently being replaced by a superior test, the ImmunoCAP.[2] The SPT is the gold standard in vivo test to detect causative allergens, but it is inconvenient and time-consuming. Additionally,

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its results can be affected by various factors such as skin diseases, and recent drug use.[3] MAST is an in vitro test that can quantify numerous allergen-specific IgEs in a small volume of serum. Unlike the SPT, results of MAST are not influenced by recent drug use. Its sensitivity and specificity, however, has been reported to be $\leq$80%, which is significantly lower than that of the SPT.[4,5] Another in vitro test, ImmunoCAP, also measures allergen-specific IgE in sera with sensitivity and specificity approaching 90%, which is superior to that of MAST.[6,7]

The enzyme-linked immunospot (ELISpot) assay identifies specific cytokine-producing cells at the single-cell level. It is much more sensitive than the classical enzyme-linked immunosorbent assay (ELISA): it can identify as few as 3 to 5 cytokine-secreting cells per 100,000 cells.[8] In clinical settings, the interferon-γ (IFN-γ) ELISpot assay, which detects IFN-γ–producing T cells, is used for the diagnosis of tuberculosis using *Mycobacterium tuberculosis*–specific antigens.[9,10] Patients with allergic rhinitis (AR) have significantly more interleukin-4 (IL-4)-positive cells in their nasal mucosa than healthy subjects, but not IL-5–positive, IL-6–positive, or IL-8–positive cells.[11] Moreover, the concentration of IL-4 in serum decreases by long-term immunotherapy in AR patients.[12] Similarly, a significant increase in the number of allergen-stimulated IL-4–positive peripheral blood mononuclear cells (PBMCs) or the IL-4 level in serum was previously identified in patients with atopic asthma compared to nonatopic asthma patients or healthy individuals.[13,14] In this study, we investigated the sensitivity, specificity, and positive and negative predictive values of the IL-4 ELISpot assay as a novel diagnostic test for sensitization to house dust mites (HDMs) in rhinitis patients.

Patients and methods

Subjects and samples

Among patients aged 20 to 59 years who underwent septoturbinoplasty and/or endoscopic sinus surgery in Gyeongsang National University Hospital, consecutive 84 patients who received the SPT for HDM were included. Blood was taken during surgery and serum and PBMCs were separated for MAST and IL-4 ELISpot assay, respectively. PBMCs were isolated by density-gradient centrifugation using lymphocyte separation medium (PAA Laboratories, Pasching, Austria) and cryopreserved in liquid nitrogen until use. Patients with diabetes mellitus or immunologic diseases other than allergy were excluded. Subjects who had received systemic corticosteroids or immunotherapy within 2 months prior to the surgery were also excluded. Subjects were categorized into HDM AR (n = 43) and control (n = 41) groups according to the SPT results. All HDM AR patients possessed at least 1 of the following symptoms: nasal stuffiness, sneezing, watery rhinorrhea, and nasal itching during 2 or more consecutive days for more than 1 hour on most days. No patients received immunotherapy or immunosuppressive drugs. Antihistamines were discontinued at least 1 week before the SPT. Among the 43 HDM AR patients, 37 were sensitized to both *Dermatophagoides farinae* (*Der f*) and *Dermatophagoides pteronyssinus* (*Der p*) (Table 1). This study was conducted according to the Declaration of Helsinki principles and approved by the institutional review board of Gyeongsang National University Hospital (IRB number: 2013-06-018). A written informed consent was received from the participants prior to inclusion in the study.

SPT for the diagnosis of HDM AR

The SPT was performed with *Der f* and *Der p* extracts, negative (saline) and positive control (histamine) solutions (Allergopharma, Reinbek, Germany). After applying each solution onto the forearm, epidermal pricks were performed. After 20 minutes, a wheal size equal or greater than that of the histamine prick was assessed as positive.

MAST

MAST was performed with AdvanSure® Allergy Screen (LG Life Sciences, Daejeon, Republic of Korea). The test procedure was performed according to the manufacturer's instructions. Briefly, 250 μL of patient serum was added to an allergen-bound nitrocellulose membrane at room temperature for 30 minutes. After washing, a biotin-conjugated antibody against human IgE was added, followed by incubation at room temperature for 30 minutes. After washing, streptavidin-conjugated alkaline phosphatase was added, followed by incubation at room temperature for 20 minutes. After additional washing, the luminescent reagent was added, followed by incubation at room temperature for 20 minutes. The test strips were then completely dried and read. The software determined the class (0 to 6) of the specific IgE concentration.

IL-4 ELISpot assay

Ninety-six–well ELISpot plates (EMD Millipore, Billerica, MA) were coated with 4 μg/mL capture antibodies for IL-4 (BD Biosciences, San Diego, CA) at 4°C overnight. Duplicate cultures of 300,000 PBMCs per well were set up in the plates. PBMCs were stimulated with 10 μg/mL *Der f* or *Der p* extracts (Greer, Lenoir, NC). Phosphate-buffered saline (PBS) and 1 μg/mL phytohemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO) were used as negative and positive control stimulants, respectively. After culture for 48 hours, 3 μg/mL of biotinylated antibodies for IL-4 (BD Biosciences) were applied. The spots were developed using the Alkaline Phosphate Conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA), and the developed spots were counted with an ELISpot reader (C.T.L., Cleveland, OH). The number of background spots in the PBS-added wells was counted. The HDM extract-stimulated spot number was calculated by subtracting the spot number in PBS-added wells from that in HDM extracts–stimulated wells. Each sample was duplicated or tripled to secure the accuracy of the procedure, and a mean value was obtained. All

TABLE 1. Characteristics and basic outcomes of the IL-4 ELISpot assay and MAST of the study participants

	HDM AR (n = 43) (*Der f*: = 39, *Der p*. = 41)	Control (n = 41)	*p*
Age (years), median (range)	27 (12–56)	34 (14–58)	0.015
Sex, n (%)			0.600
Male	35 (47.0)	31 (53.0)	
Female	8 (55.6)	10 (44.4)	
Number of background spots, median (range)	9.7 (5.0–17.0)	11 (7.8–18.3)	0.128
Der f (IL-4 ELISpot), median (range)	23.3 (0.0–87.5)	0.0 (0.0–10.8)	<0.001
Der p (IL-4 ELISpot), median (range)	17.5 (0.0–59.2)	0.0 (0.0–8.3)	<0.001
Der f (MAST), median (range)	4.0 (0.0–6.0)	0.0 (0.0–6.0)	<0.001
Der p (MAST), median (range)	4.0 (0.0–6.0)	0.0 (0.0–6.0)	<0.001

AR = allergic rhinitis; *Der f* = *Dermatophagoides farinae*; *Der p* = *Dermatophagoides pteronyssinus*; ELISpot = enzyme-linked immunospot; HDM = house dust mite; IL = interleukin; MAST = multiple allergen simultaneous test.

the procedures were done by C.D.Y. who was blinded to the SPT results of each subject.

Statistical analyses

Statistical analyses were performed using SPSS ver. 21.0 for Windows (IBM Corp., Armonk, NY) and MedCalc Statistical Software ver. 15.2.2 (MedCalc Software bvba, Ostend, Belgium). Mann-Whitney *U* test was used to compare age, MAST class, and the number of IL-4 spots between AR patients and control subjects since no variables satisfied the assumption of normal distribution and equal variances by Shapiro-Wilk test and Levene's test. Fisher's exact test was used to compare the gender ratio between groups. The receiver operating characteristic (ROC) curve analysis was conducted for the IL-4 ELISpot assay and MAST results; the maximum value of Youden's index (sensitivity + specificity − 1) was determined as an optimal cutoff value, the SPT being considered the gold standard test. Sensitivity, specificity, positive predictive value, and negative predictive value were compared between the IL-4 ELISpot assay and MAST results based on the cutoff value determined (as described in the previous sentence). To determine the superiority in the efficiency of the tests, the areas under the curve (AUCs) were compared. A p value <0.05 was considered to indicate statistical significance.

Results

Comparison of IL-4 ELISpot assay results between subject groups

The median numbers of background spots were 9.7 (range, 5.0-17.0) and 11 (range, 7.8-18.3) in HDM AR patients and control subjects, respectively ($p = 0.124$) (Table 1; Fig. 1A). In contrast, the median number of *Der f*–stimulated IL-4 spots was significantly higher in samples from *Der f*–positive AR patients than those from control subjects ($p < 0.001$; Table 1; Fig. 1B, D). Similarly, the median number of *Der p*–stimulated IL-4 spots was significantly higher in samples from *Der p*–positive AR patients than those from control subjects ($p < 0.001$; Table 1; Fig. 1C, D).

Comparison of the IL-4 ELISpot assay and MAST for diagnosis of HDM allergy

In the ROC curve analysis, optimal cutoff values of the IL-4 ELISpot assay were 6.7 for *Der f* and 2.0 for *Der p*, respectively; those of MAST were class 1 for *Der f* and class 2 for *Der p* (Table 2). Based on these cutoff values, sensitivity and specificity of the IL-4 ELISpot assay were significantly higher than those of MAST for both *Der f* and *Der p* (Table 2). Accordingly, positive and negative predictive values of the IL-4 ELISpot assay were also significantly higher than those of MAST for both *Der f* and *Der p* (Table 2). The AUCs of the IL-4 ELISpot assay and MAST for *Der f* were 0.939 (range, 0.864-0.979) and 0.777 (range, 0.673-0.860), respectively (Fig. 2A). For *Der p*, the AUCs of the IL-4 ELISpot assay and MAST were 0.971 (range, 0.908-0.995) and 0.788 (range, 0.685-0.869), respectively (Fig. 2B).

Discussion

The ELISpot assay was introduced in 1983 for enumeration of antibody-secreting cells, and was modified thereafter to assay levels of antigens (ie, cytokines) released by cultured cells.[15,16] As few as 10 to 100 cells per well were sufficient for the detection of cytokine-releasing cells using ELISpot, whereas at least 1×10^4 cells were required for cytokine detection by ELISA.[17] Similarly, in another study, an IL-4 ELISpot assay displayed a higher sensitivity for detection

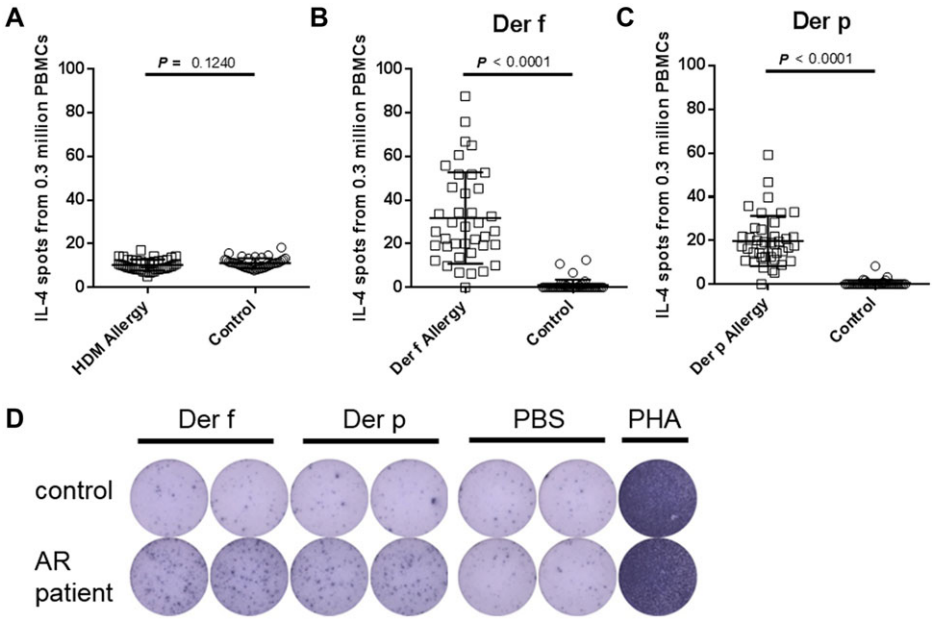

FIGURE 1. IL-4 ELISpot assay results. (A) Comparison of the background spot numbers between HDM AR patients (n = 39 for *Der f*; n = 41 for *Der p*) and control subjects (n = 45). (B) Comparison of *Der f*–stimulated IL-4 spot numbers between groups. (C) Comparison of *Der p*–stimulated IL-4 spot numbers between groups. Data is presented as mean and SD. Plotted values in B and C are data from which the mean background spot numbers have been subtracted. (D) Representative IL-4 ELISpot assay results. AR = allergic rhinitis; *Der f* = *Dermatophagoides farina*; *Der p* = *Dermatophagoides pteronyssinus*; ELISpot = enzyme-linked immunospot; HDM = house dust mite; IL-4 = interleukin-4; PBMC = peripheral blood mononuclear cell; PBS = phosphate buffered saline; PHA = phytohemagglutinin; SD = standard deviation.

TABLE 2. Comparison of the accuracy of the IL-4 ELISpot assay vs MAST in the diagnosis of *Der f* and *Der p* allergy

	Optimal cutoff value	Sensitivity % (95% CI)	Specificity % (95% CI)	Positive predictive value % (95% CI)	Negative predictive value % (95% CI)
IL-4 ELISpot assay					
Der f	6.7	88.4 (74.1–95.6)	97.6 (85.6–99.9)	97.4 (84.9–99.9)	88.9 (75.2–95.8)
Der p	2.0	95.3 (82.9–99.2)	97.6 (85.6–99.9)	97.6 (85.9–99.9)	95.2 (82.6–99.2)
MAST					
Der f	1	76.7 (61.0–87.7)	73.2 (56.8–85.2)	75.0 (59.4–86.3)	75.0 (58.5–86.8)
Der p	2	69.8 (53.7–82.3)	75.6 (59.4–87.1)	75.0 (58.5–86.8)	70.5 (54.6–82.8)

Der f = *Dermatophagoides farinae*; *Der p* = *Dermatophagoides pteronyssinus*; CI = confidence interval; ELISpot = enzyme-linked immunospot; IL = interleukin; MAST = multiple allergen simultaneous test.

of IL-4–producing cells compared to ELISA or real-time reverse-transcription polymerase chain reaction. Furthermore, it was the only method that could detect spontaneous, not antigen-specific, secretion of IL-4.[18] In a study of nickel-allergic patients, ELISpot was more sensitive for detection of IL-4–producing cells compared to ELISA.[19] Due to its exquisite sensitivity, ELISpot is considered the technique of choice for detection of secretion of cytokines such as IFN-α, IFN-γ, IL-4, IL-5, IL-6, and tumor necrosis factor (TNF)-α.[20]

Several studies on the clinical applications of ELISpot have been conducted. For example, a *Mycobacterium tuberculosis* early secretory antigenic target (ESAT-6) and culture filtrate protein (CFP)-10–based IFN-γ ELISpot assay was reported to detect active tuberculosis in human immunodeficiency virus (HIV)–positive individuals.[10] It was first commercialized in the United Kingdom, named the "T-SPOT.*TB* test," and is considered to have an accuracy similar to that of the tuberculin skin test for detection of tuberculosis infection or the diagnosis of disease in children.[21] ELISpot has also been used to evaluate immune function in patients with HIV infection or cancer. In a previous study, HIV-infected patients had 4-fold fewer natural IFN-α–producing PBMCs compared to healthy subjects in response to herpes simplex virus infection.[22] In another study, an IFN-γ ELISpot assay identified an impairment of T-cell function in response to the influenza matrix peptide in melanoma patients with progressive disease.[23]

Several studies have focused on the use of ELISpot as a diagnostic tool for allergic diseases. In a previous study,

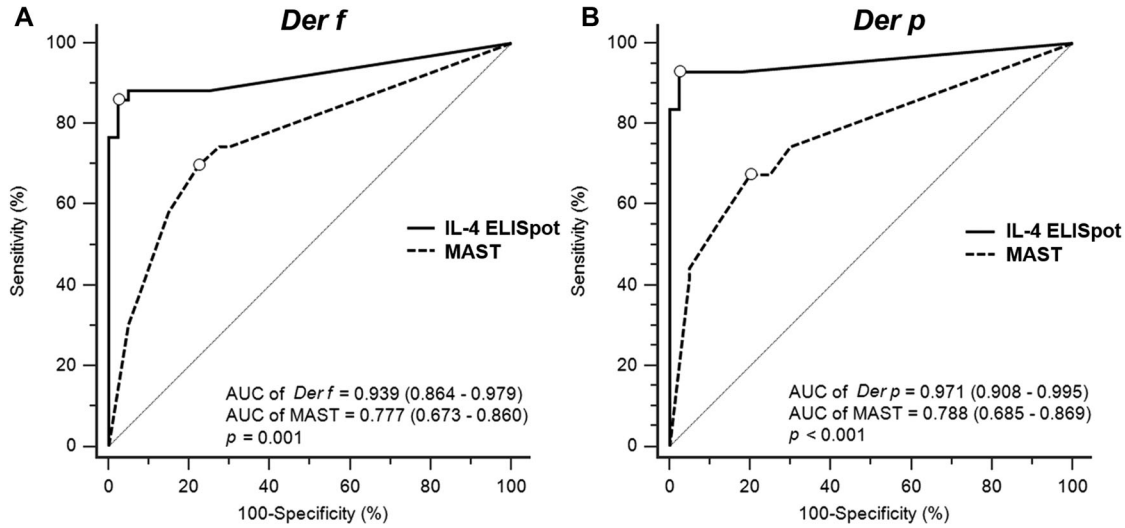

FIGURE 2. Comparison of areas under the receiver operating characteristic curve of the IL-4 ELISpot assay and MAST results for *Der f* (A) and *Der p* (B). The optimal cutoff values are marked with an open circle. AUC = area under the receiver operating characteristic curve; *Der f* = *Dermatophagoides farina*; *Der p* = *Dermatophagoides pteronyssinus*; ELISpot = enzyme-linked immunospot; IL-4 = interleukin-4; MAST = multiple allergen simultaneous test.

PBMCs were taken from healthy and atopic subjects sensitized to birch and/or cat, as identified using a RAST. Changes in the number of IL-4–producing and IFN-γ–producing cells in response to the specific allergens were evaluated by IL-4 ELISpot assay; the number of IL-4–producing cells was significantly higher in atopic asthma patients compared with healthy subjects.[13] Similarly, in another study, an increased number of IL-4–producing cells in response to cat and birch allergens were found in PBMCs from asthmatics compared with nonasthmatic subjects.[24] In another study, the correlations between the number of IL-4–producing cells measured by ELISpot assay and nasal symptoms or allergen-specific IgE levels in the blood were examined in seasonal AR patients sensitized to Japanese cedar pollen. During the pollen season, both the number of IL-4–producing cells responsive to specific allergens and the levels of cedar-specific IgE were increased.[25]

Although several previous studies have shown the feasibility of the IL-4 ELISpot assay for the detection of allergen-stimulated IL-4–producing cells, its sensitivity and specificity as a diagnostic test for AR in comparison with MAST or ImmunoCAP has not been assessed. In the present study, the specificity and sensitivity of the IL-4 ELISpot assay were superior to that of MAST for HDM allergen detection, suggesting that the IL-4 ELISpot assay is superior to MAST for the diagnosis of HDM AR. Like MAST, IL-4 ELISpot assay can handle large numbers of samples and detect many allergens simultaneously. Thus, if this assay can also accurately detect additional allergen (ie, pollens, fungi, animal dander, or insects)-stimulated IL-4–producing cells, it may be a useful in vitro test for the diagnosis of AR or other allergic diseases.

One of the drawbacks of the IL-4 ELISpot assay is nonspecific background spots. It is necessary to reduce the number of background IL-4 spots and increase the number of IL-4 spots in allergen-stimulated wells. As reported previously, wrapping the plates with regular aluminum foil is a simple method of reducing the number of background spots.[26] Use of synthetic peptide as a stimulant is an alternative method. HDM extracts contain nonprotein endotoxins that might induce allergen-nonspecific cytokine secretion.[27,28] Immunodominant peptide regions from HDM proteins can be selected and synthesized in vitro. The number of background IL-4 spots might be reduced by stimulating subjects' PBMCs with synthesized peptides. Additionally, the number of allergen-stimulated IL-4 spots can be increased using concentrated immunodominant peptides rather than whole HDM proteins. Because granulocytes such as basophils and neutrophils can also produce IL-4, in addition, the number of background IL-4 spots may be reduced by removing granulocytes during PBMC separation.[29,30] By using the abovementioned methods, further study will be needed to reduce the number of background spots. In doing so, the positive criteria for the IL-4 ELISpot assay can be modified, resulting in the elevation of sensitivity of the IL-4 ELISpot assay. Another drawback of the IL-4 ELISpot assay is that it is relatively labor-intensive and requires live cells. It is inferior to the MAST or ImmunoCAP in regard to convenience in the sample storage. Furthermore, the cost can be another issue. Except for personnel expenses, in the present study, it cost approximately $12 to conduct IL-4 ELISpot assay for *Der f* and *Der p* per each subject. By testing multiple allergens simultaneously, however, costs per allergen would be lowered.

Besides abovementioned disadvantages of the IL-4 ELISpot assay, this study has several limitations. First, the ImmunoCAP has superior sensitivity and specificity to the MAST.[7] Instead of the MAST, therefore, the ImmunoCAP should have been used as a control test to enhance the clinical value of the IL-4 ELISpot assay. Because the

ImmunoCAP was not available in our institution, however, that could not be done. This study is also limited by an uneven age distribution between subject groups: average age in the late twenties (HDM AR patients) vs midthirties (control subjects) (Table 1). However, as both groups were young adults aged 20 to 40 years, the difference in average age likely did not have significant effects on the results. The other limitation is the possibility of allergen-nonspecific production of IL-4 in response to HDM allergen stimulation. To obviate this possibility, the IL-4 ELISpot assay should have also been done after stimulation of other common allergens such as cat or dog in PBMCs from the subjects who show negative SPT results to them. Furthermore, to make a confirmative diagnosis for HDM allergic rhinitis, nasal provocation tests should have been conducted. However, given that HDM is a predominant causative allergens and less than 8% of AR patients are sensitized to other perennial allergens such as cockroach, dog, cat, and fungus in Korea, it is not likely that other allergens are true causative allergens for patients sensitized to HDM.[31]

Conclusion

The IL-4 ELISpot assay showed superior specificity, sensitivity, and positive and negative predictive values to MAST for identification of the causative allergens in patients with HDM AR. Further research, such as confirmation of the accuracy of the IL-4 ELISpot assay for allergens other than HDM, will be needed to confirm its usefulness in diagnosis of sensitization toward specific allergens in patients with AR.

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