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Microarray-based evaluation of selected recombinant timothy grass allergens expressed in *E. Coli* and *N. Benthamiana*

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Abstract

Background Timothy grass (Phleum pratense) is a significant source of allergens, and recombinant allergens are increasingly used for diagnostic purposes. However, the performance of different recombinant allergen production systems in diagnostic assays needs further investigation to optimize their use in clinical settings.

Objective The main objective of this study was to analyze and compare the diagnostic performance of recombinant timothy grass allergens produced in *E. coli* and *N. benthamiana* using a custom-made microarray chip.

Methods Recombinant timothy grass allergens Phl p 1, Phl p 2, Phl p 5, Phl p 6, Phl p 11, and Phl p 12 were produced in *E. coli* and/or *N. benthamiana*. A total of 113 patient serum samples were tested to evaluate the diagnostic sensitivity, specificity, inter-assay variability, and correlation of allergen-specific IgE detection compared to commercial multiplex tests (ALEX and ISAC). Additionally, the prevalence of sIgE to these allergens was assessed.

Results Phl p 1, Phl p 2, Phl p 5, Phl p 6 and Phl p 11 showed high or very high positive correlation in immunoreactivity with other commercial multiplex tests. Notably, Phl p 11 fused with maltose-binding protein (MBP) demonstrated high diagnostic specificity and sensitivity, with a 0.3 arbitrary cut-off value. However, a high intra-assay variation was observed. The study also assessed specific IgE prevalence to timothy grass allergens within the tested patient cohort.

Conclusions Recombinant allergens from both *E. coli* and *N. benthamiana* demonstrated strong diagnostic potential on the microarray platform, with Phl p 11 (MBP-fused) showing particularly high performance. High intra-assay variation highlights the need for further optimization in allergen formulation and microarray storage conditions. These results highlight the potential of recombinant allergens for diagnostic applications, despite challenges with allergen stability in microarray formats. Specific IgE prevalence to timothy allergens revealed a sensitization profile consistent with findings from multiple studies.

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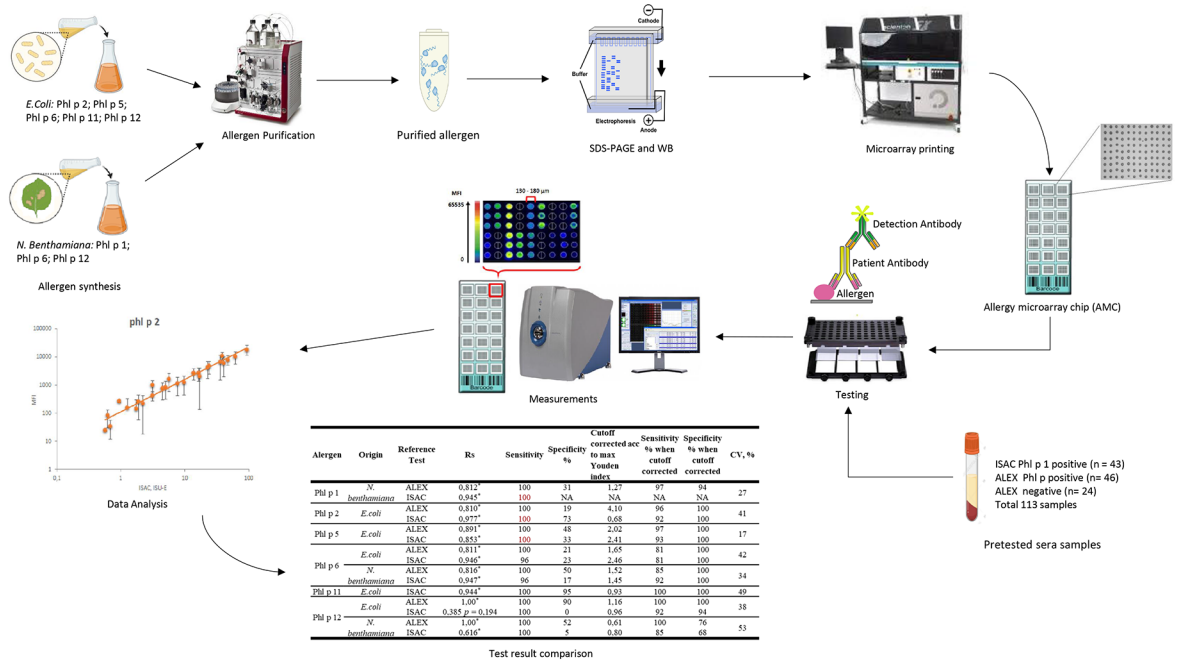
Graphical Abstract



Alergen	Origin	Reference Test	Rs	Sensitivity	Specificity %	Cutoff corrected acc to max Youden index	Sensitivity % when cutoff corrected	Specificity % when cutoff corrected	CV, %
Phl p 1	*N. benthamiana*	ALEX	0,812*	100	11	1,27	97	94	27
		ISAC	0,945*	100	NA	NA	NA	NA	
Phl p 2	*E.coli*	ALEX	0,810*	100	19	4,10	96	100	41
		ISAC	0,977*	100	73	0,68	92	100	
Phl p 5	*E.coli*	ALEX	0,891*	100	48	2,02	97	100	17
		ISAC	0,853*	100	33	2,41	93	100	
Phl p 6	*E.coli*	ALEX	0,811*	100	21	1,65	81	100	42
		ISAC	0,946*	96	23	2,46	81	100	
	N. benthamiana	ALEX	0,816*	100	50	1,52	85	100	34
		ISAC	0,947*	96	17	1,45	92	100	
Phl p 11	*E.coli*	ISAC	0,944*	100	95	0,93	100	100	49
Phl p 12	*E.coli*	ALEX	1,00*	100	90	1,16	100	100	38
		ISAC	0,385 p = 0,194	100	0	0,96	92	94	
	N. benthamiana	ALEX	1,00*	100	52	0,61	100	76	53
		ISAC	0,616*	100	5	0,80	85	68	

Keywords Recombinant allergen, Allergen microarray, Timothy grass pollen, Maltose-binding protein

Introduction

The IUIS Allergen Nomenclature Sub-Committee has officially recognized 10 timothy grass allergens: Phl p 1, Phl p 2, Phl p 3, Phl p 4, Phl p 5, Phl p 6, Phl p 7, Phl p 11, Phl p 12, Phl p 13. Three allergen components of timothy grass demonstrate cross-reactivity with pollen sources unrelated to this grass and belong to pan-allergen protein families including polcalcins Phl p 7, profilins Phl p 12, and Ole e 1-related proteins Phl p 11 [1]. Among all, the beta-expansin Phl p 1 is the most prevalent *Phleum pratense* allergen and is considered a significant primary marker of grass pollen sensitization [1]. However, the absence of sensitization to Phl p 1 does not exclude the possibility of true grass pollen sensitization. Phl p 2 exhibits structural similarities to beta-expansins [2]. Phl p 2 is also a major allergen, potentially serving as a secondary marker for grass pollen sensitization [1] Another major allergen, Phl p 5, is also an essential marker for grass pollen sensitization. Monosensitization to Phl p 5 is rare [3]. Sensitization to Phl p 5 usually develops at a later stage than Phl p 1 and is subsequently followed by Phl p 2, Phl p 6 and Phl p 11. Phl p 6, which shares a high structural resemblance to Phl p 5, displays overlapping epitopes on the N-terminal domain [4], leading to high cross-reactivity with Phl p 5.

Many allergens originate from plants and may have plant-specific post-translational modifications that are important for IgE recognition, thus utilizing plant expression systems for synthesizing recombinant allergens stands as a prominent application, particularly in producing allergens for diagnostic purposes. Plants offer several advantages over bacterial expression systems, such as the ability to perform complex eukaryotic post-translational modifications (disulfide-bond formation or glycosylation), which are essential for producing biologically active recombinant allergens with IgE binding capacities identical to those of their natural counterparts [5]. However, only a few allergens have been successfully expressed in plants [6]. This may be due to the technical challenges and expertise required to establish plant-based expression systems, compared to bacterial or yeast systems. Different expression systems and production methods can alter protein structure, introduce specific modifications, and thereby affect the reactivity of recombinant allergens with IgE. For instance, Phl p 1 expressed in insect cells had superior IgE recognition when compared to Phl p 1 from *E. coli* [7]. However, the synthesis of recombinant Phl p allergens in plants has not yet been explored.

Affinity tags frequently used in the purification of recombinant proteins may also potentially impact the folding of the proteins, thus influencing their immunological and allergenic properties. For instance, when Phl p 1 was expressed in *E. coli* as non-fused his tagged

allergen, inclusion bodies formed [8–10]. Among the various fusion tags, Maltose Binding Protein (MBP) stands out as a popular choice due to its substantial size, weighing around 43 kDa. Studies indicate that MBP not only enhances the expression levels and solubility of the target protein but also has a beneficial effect on allergen production [11]. Usually, the fusion tags are cleaved for further analysis [12], but in previously published studies [13, 14] studies MBP fused allergens, did not interfere with immunochemical assay outcomes, showing that MBP is an excellent candidate as a fusion partner in the manufacturing of recombinant allergens .

Microarray technology possesses the potential to revolutionize allergy diagnostics by enabling simultaneous assessment of specific IgE against multiple target proteins, utilizing minimal patient serum volume [1, 15–19]. These microarrays, encompassing various common environmental and food allergen components, empower clinicians to gain a comprehensive perspective on the patient's specific IgE reactivity profile [2, 3, 15, 20–24].

Despite the claims of various test manufacturers that their specific IgE (sIgE) tests are aligned with the WHO standard, there is considerable evidence [25] that sIgE measurements are not consistent across different platforms. Different sIgE tests, such as ImmunoCAP singleplex and ISAC (Thermo Fisher Scientific, Sweden), use different technologies to detect the presence of sIgE antibodies in the patient blood. Thus, the results of sIgE tests are not interchangeable, as they may differ significantly depending on the method and the allergen [26–28]. Therefore, there is no simple way to convert the values obtained from one test to another.

In this study we aimed to analyze recombinantly produced timothy grass allergens (Phl p 2; Phl p 5; Phl p 6; Phl p 11; Phl p 12) from *E. coli* as MBP fused variants and (Phl p 1; Phl p 6; Phl p 12) from *N. benthamiana* expression systems for their diagnostic capabilities to determine the quantity of IgE using microarray technology. Thus other muliplex micrroaray assays (ISAC and ALEX) were used as reference test for performance comparison. The selection of Phl p 1, Phl p 2, Phl p 5, Phl p 6, Phl p 11, and Phl p 12 for this study was influenced by primarily the successful production and purification of recombinant allergens in *E. coli* and/ or *N. Benthamiana.*

Materials and methods

Allergen identification, cloning, expression and purification of Phl p 1, Phl p 6, and Phl p 12 in *N. benthamiana*

Codon optimization was deemed unnecessary for expression in *N. benthamiana*, as plant expression systems are generally capable of processing native sequences from other plant species without requiring optimization. All allergen coding sequences for plant expression were obtained from timothy grass pollen RNA. Timothy flower heads were collected during flowering season in the surroundings of Vilnius, Lithuania. RNA was extracted using Spectrum Plant Total RNA Kit (Sigma, USA), and cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) as described previously [29]. Coding sequences of *P. pratense* allergens Phl p 1, Phl p 6, and Phl p 12 were amplified using primers presented in supplementary material 3. Tables, sequenced, and inserted into MagniIcon vector [30] modified to add 6xHis-tags to the N-termini of expressed proteins. Since Phl p 1 and Phl p 6 are naturally secreted proteins, their His-tagged coding sequences were also fused to rice alpha-amylase signal sequence. Sequence analysis revealed that cloned allergens (NCBI GenBank accession numbers **OR596406**, **OR596409**, **OR596411**) were identical to or closely resembled allergen variants Phl p 1.0101, Phl p 6.0102, and Phl p 12.0102 (supplementary material 3. Tables).

For transient expression, *N. benthamiana* plants were grown five to six weeks at 22 °C with a 16 h light and 8 h dark photoperiod and vacuum-infiltrated with *A. tumefaciens* strain GV3101 transformed with expression vectors. Six to seven days after infiltration, leaves were collected, grinded in liquid nitrogen, and soluble proteins extracted by applying 5 ml of buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF) to 1 g of tissue. Cell debris was removed by centrifugation at 40,000 × *g* for 25 min at 4 °C. The supernatant was filtered through Millex-HV filter with a 0.45 µm pore size hydrophilic PVDF membrane (Merck Millipore, UK).

Purification of recombinant proteins was carried out using ÄKTA purifier 100 chromatography system equipped with the sample pump P-960 and the fraction collector Frac-920 (GE Healthcare Bio-Sciences AB, Sweden) at 4 °C. Chromatographic purification was performed using 1 ml HisPur™ Ni-NTA Chromatography Cartridge (Thermo Fisher Scientific, USA) at a flow rate of 1 ml/min. Ni-NTA cartridge was equilibrated with 10 column volumes (c.v.) of equilibration/binding buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole). The filtered leaf extract was loaded onto Ni-NTA cartridge. The cartridge was then washed with 12–15 (c.v.) of wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole). Elution was performed with 6–7 (c.v.) of elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 300 mM imidazole). Protein elution was monitored by absorbance at 280 nm. After chromatography, protein samples were analyzed by SDS-PAGE. Selected elution fractions were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 7.4 at 4 °C. After dialysis, the protein solution was centrifuged at 15,000 × *g* for 30 min at 4 °C.

Cloning, expression, and purification of recombinant allergens Phl p 2, Phl p 5.02, Phl p 6, Phl p 11, Phl p 12 in *E. coli*

Synthetic genes with optimized codons for *E. coli* were used for bacterial *E. coli* expression in order to ensure efficient transcription and translation in bacterial host. The coding sequences of allergens Phl p 2.0101, Phl p 5.0201, Phl p 6.0101, Phl p 11.0101, and Phl p 12.0101 were obtained from GenBank (accession no. **X75925**, **Z27083**, **Z27082**, **AF521563** and **X77583** respectively). The codon-optimized allergen gene sequences (GenBank accession no. **OR634925**, **OR634928**, **OR634926**, **OR634927** and **OR634924** respectively), were synthesized by General Biosystems in Morrisville, USA. The gene sequence was delivered in the pUC57 cloning vector, with BamHI and XhoI restriction endonuclease cutting sites flanking the gene sequence.

For the expression of recombinant timothy allergens in *E. coli*, the recombinant plasmid pUC57 containing the Phl p allergen DNA sequence was used. Gene sequences mentioned earlier were excised from the plasmid using BamHI and XhoI restriction endonucleases. Each gene was cloned into a previously digested pET28-MBP-TEV vector, which was kindly provided by Zita Balklava and Thomas Wassmer (RRID: Addgene_69929) [31]. The resulting construct contained the respective Phl p allergen encoding gene fused to the MBP encoding sequence.

The selected recombinant plasmid was introduced into *E. coli* BL21(DE3) cells through a heat-shock transformation method. The growth of the *E. coli* culture and the expression of the recombinant protein were carried out with some modifications based on the manufacturer's recommendations (Novagen, Merck, USA). The synthesis of Phl p allergens was induced by adding 0.25 mM IPTG (Sigma Aldrich, Germany). The cells were then cultivated 2.5 h at 37 °C. Subsequently, the cells were harvested and disrupted using sonication (Bandelin Sonopuls HD 3100, Bandelin Electronic, Germany). The soluble and insoluble fractions were separated through centrifugation at 13.200 g for 20 min at 4 °C and were analyzed using SDS-PAGE under reducing conditions.

Approximately 0.5 g of bacterial cells were vortexed in 3 ml of HIS binding buffer (100 mM Tris–HCl, 500 mM NaCl, 20 mM imidazole, 1 mM PMSF, pH 8). The cell suspension was sonicated (5 min;15 s disruption, 15 s cooling at 20 kHz and 60%). Following sonication, the lysate was divided into two 2 ml tubes and then centrifuged at 20,000 g for 20 min. The resulting supernatant was filtered through a 0.22 µM non-sterile PES membrane and subsequently loaded onto a HisTrap HP 1 mL column (Cytiva, Sweden) for Ni^{2+} affinity chromatography. Elution was carried out with a HIS elution buffer (100 mM Tris–HCl, 500 mM NaCl, 500 mM imidazole, 1 mM PMSF, pH 8).

The collected fractions were pooled and then desalted using a HiPrep 26/10 Desalting column (Cytiva, Sweden) into MBP binding buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4). Subsequently, the eluate was applied to an MBPTrap HP 1 mL column (Cytiva, Sweden) and eluted with a 10 mM maltose solution in the MBP binding buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM maltose, 1 mM EDTA, pH 7.4). Both chromatography steps were performed using an Äkta Avant system (Cytiva, Sweden). The purity of the final product was assessed using SDS-PAGE.

The concentration of purified protein was determined by Roti-Quant (Bradford) assay (Carl Roth, Germany). A BSA pre-diluted set (Thermo Fisher Scientific Baltics, Lithuania) was used to determine the calibration curve.

SDS-PAGE and immunoblot analysis

As a positive control, MBP expressed and purified from *E. coli* was used [14]. As a negative control, 2 mg/ml BSA standard (Thermo Fisher Scientific Baltics, Cat. No. 23208, Lithuania) was used. The protein samples were mixed with the 2× reducing protein loading buffer (0.5 M Tris-HCl pH 6.8, 4% (w/v) SDS, 0.1% bromophenol blue, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol), heated for 10 min. at 100 °C and 0.3 µg of each protein was loaded into wells of 14% SDS-PAGE gel. Proteins were fractionated in gel electrophoresis in SDS-Tris-glycine buffer (Carl Roth, Germany), using Mighty Small™ Mini Vertical Electrophoresis system (Hoeferca, USA). After electrophoresis, polyacrylamide gels were stained with Coomassie Brilliant Blue dye solution (0.5% Coomassie brilliant blue R-250, 50% ethanol, 10% acetic acid) or proteins were transferred onto 0.2 µm nitrocellulose membrane (GE Healthcare, USA) for immunoblot analysis under semi-dry conditions using Semi-Dry Blotter (Novex Invitrogen, US). After protein transfer, membrane was incubated in fixation solution (80 mM Na_2HPO_4, 25 mM $NaH_2PO_4 \times H_20$, 100 mM NaCl, 4% formaldehyde) for 30 min. at room temperature (RT). After fixation, the membrane was washed several times with blot wash solution (80 mM Na_2HPO4, 25 mM $NaH_2PO_4 \times H_20$, 100 mM NaCl, 0.1% Tween-20). Then, membrane was blocked with 1× Roti-Block solution (Carl Roth, Germany) for 1 h at RT with shaking. After several washes with PBS-T solution, membrane was incubated for 1 h with anti-Tetra His antibodies (1:1000 in PBS-T, QIAGEN, Cat. No. 34670, Germany). The membrane was washed several times with blot wash solution and incubated for 1 h with HRP-conjugated anti-mouse IgG antibody (1:3000 in PBS-T, BioRad, Cat.No. 1721011, USA). After several washes with blot wash solution, membrane was washed with distilled water and stained with PierceTM 1-Step Ultra TMB blotting solution (Thermo Fisher Scientific Baltics, Cat. No. 37574, Lithuania).

Allergy microarray chip development

Timothy allergens were printed as a series of dilutions (1, 2, 4, 8, 16 and 32 times) starting from stock concentration, onto 2D-Epoxy glass slides (PolyAn GmbH, Germany) using the sciFLEXARRAYER SX microarray printer (Scienion GmbH, Germany). Each allergen was printed as a single droplet (400–420 pL/ drop) in three replicates at a spot-to-spot distance of 330 µm. Streptavidin-Cy5 (SouthernBiotech, USA) were printed as guide dots (GD) for grid alignment. For assay control purposes a number of proteins were spotted at 0.1 mg/ml concentration: native human IgE antibody (Abcam, Cat. No. AB65866, UK) – detection reagent control; MBP – control for unspecific binding to recombinant allergens fused with MBP; HRP (Sigma Aldrich, Cat. No. 77322, Germany) – positive control to IgE antibodies cross-reacting to carbohydrate determinants (anti-CCD IgE); printing buffer (PBS pH 7,4) (PB) – spot background control. The arrays were stored overnight at 4°C prior to use. Before the experiment, the slides were dried at 37 °C for 15 min. Blocking was carried out using Super G blocking buffer (Grace Bio Labs, USA) and gently stirring the slides for 15 min at RT. After washing once for 30 s with deionized water, the slides were dried via centrifugation for 10 s. Subsequently the slides were fitted into 24-Well Hybridization Cassette (Arrayit Corporation, CA, USA) and 80 µL of sera, diluted five times with dilution buffer (2% BSA in PBS-T) was added to each well. The fitted slides were sealed with an adhesive seal strip and incubated for 2 h at 37°C. After washing five times with 100 µL/well of PBS-T, the slides were incubated with the detection reagent (1 µg/ml of mouse anti-human IgE Alexa Fluor 647 conjugate (Institute of Biotechnology, Vilnius University) diluted in dilution buffer) at 80 µL/well for 30 min. at 37°C. After incubation, the slides were washed five times with 100 µL/well of PBS-T. After removing the 24-Well Hybridization Cassette, the slides were incubated with PBS-T with gentle agitation for 10 min. After the final wash for 30 s with deionized water, the slides were dried via centrifugation for 10 s. Slides were scanned using the InnoScan 710 AL microarray scanner (Innopsys, France) with photomultiplier tube (PMT) settings 40, at 635 nm wavelength. The images were analyzed with the MAPIX software (Innopsys, France). The averaged median fluorescence intensity (MFI) and ALEX or ISAC sIgE concentration values for the chosen optimal dilution were used to calculate calibration curve parameters for each allergen.

sIgE measurements

ImmunoCAP ISAC 112 (ThermoFisher Scientific, Sweden) is a solid-phase immunoassay that enables the simultaneous measurement of sIgE against 112 molecular components from 51 different allergenic sources. The assay was performed according to the manufacturer's instructions. In brief, glass slides were pre-washed with washing solution for 10 min, then rinsed with distilled water for 30 s and dried for 15 min. Next, 30 µl of undiluted serum samples were applied to each microarray and incubated for 2 h at RT. After washing, rinsing and drying again, 30 µL of fluorescence-labeled anti-human IgE antibodies were added and incubated for another 30 min at room temperature. Finally, the slides were washed, rinsed and dried once more and scanned using a fluorescence scanner (excitation wavelength 532 nm). The fluorescence signals from the anti-IgE antibodies were compared with a calibration curve and expressed as arbitrary ISU-E units.

ALEX chip is a of solid-phase type immunoassay (MacroArray DX, Vienna, Austria) that includes 157 allergen extracts and 125 molecular components. These allergens and components are arranged on a nitrocellulose membrane in a cartridge. The assay was conducted following the manufacturer's instructions. In summary, chip was incubated with 0.5 ml of 5 times diluted serum sample under mild agitation. After 2 h of incubation, the chips were thoroughly washed, and a detection reagent of anti-human IgE conjugated with alkaline phosphatase was applied and incubated for 30 min. After another round of thorough washing, the enzyme substrate was added, and the reaction was completed in 8 min. The membranes were dried, and the color reaction intensity for each allergen spot was captured by a CCD camera. The software processed the images and generated a report that showed the allergens and components and their concentration in kUa/L.

Sera samples

Inclusion criteria for the study were a serum level of IgE specific to timothy grass pollen extract (*Phleum pratense*)≥0,3 kUa/L for ALEX (Macro ArrayDX, Austria) and Phl p 1≥0,3 ISU-E for ISAC test. The later test results were included in this study to compare Phl p 11 diagnostic performance. A total of 43 ALEX and 46 ISAC positive serum samples were selected. Additionally, 24 serum samples confirmed as negative by ALEX test were used to assess the specificity of recombinant timothy allergens. Serum samples were collected in Santaros Clinical Hospital and stored at −20 °C after blood collection until analysis by microarray assays. Lithuanian Bioethics Committee for Biomedical Research approved the study protocol (Nr. 158200–17–926–430). All participants provided their informed written consent before participating in the study. For participants who were minors, written consent was also obtained from their parents or guardians. The data were anonymized to protect the privacy and confidentiality of the participants.

Statistical analysis

To determine inter-assay measurements variability of allergy microarray assay (AMC) positive serum samples were re-tested after 7 weeks. The coefficient of variation (CV) was calculated as the percentage of the standard deviation of the two averaged MFI values divided by the average of averaged MFI values. The lowest (0) and highest (65535) MFI values were excluded from calculations. For the calculations of sensitivity and specificity of the AMC test, the ISAC or ALEX measured values of sIgE in serum samples were used as reference. Youden index was calculated to determine the optimal cut-off value to achieve the highest specificity and sensitivity for a particular allergen [32]. The correlation of the sIgE levels measured by AMC and positive reference microarray tests result values (ALEX or ISAC) was depicted by scatterplots. The Spearman's Correlation coefficient (Rs) was calculated. Spearman's rho values were categorized as very high positive correlation (0.9–1.0), high positive (0.7–0.9), moderate positive (0.5–0.7), low positive (0.3–0.5) or negligible (below 0.3). Differences of p less than 0.05 were statistically significant. Correlation analysis and graphs were performed in GraphPad Prism 9.0 (GraphPad Software). CV, sensitivity, specificity and Youden index were calculated using Excel 2016, Microsoft Office (Microsoft).

Results and discussion

Prevalence of sensitization to timothy grass allergens

The sensitization prevalence to timothy allergens according to ISAC results ($n=46$) were 100% to Phl p 1; 61% - Phl p 2; 70% - Phl p 5; 61% - Phl p 6; 26% - Phl p 11; and 28% to Phl p 12. A comparable patient sensitization profile was observed when another group of samples ($n=43$) were tested by ALEX assay: 86% - Phl p 1; 56% - Phl p 2; 79% - Phl p 5; 63% - Phl p 6; 12% - Phl p 12 (supplementary material 3. Tables). A more than twofold higher prevalence of sensitization to Phl p 12 was observed with ISAC samples. This might be associated with the difference in sera inclusion criteria for this study. ISAC samples were all selected to be positive to Phl p 1, while ALEX sample inclusion criteria was timothy extract positive samples. Among 43 timothy extract positive serum samples 6 were negative to Phl p 1, but were positive to Phl p 5, indicating that Phl p 1 is not always the primary sensitizing molecule. Thus quantification for sIgE against Phl p 5 must be carried out to improve diagnostic accuracy.

In one study 183 sera were tested with ImmunoCAP from individuals allergic to grass pollen from different parts of Europe, Canada, and Japan [33]. They found that Phl p 1 and Phl p 5 were the most prevalent allergens, with 88% and 71%, respectively. And Phl p 2 was detected in sera at a lower frequency, with a detection rate of 46%. Similar results were found in a study led by Sekerkova where 276 patients allergic to timothy grass pollen were tested with ImmunoCAP for sIgE to Phl p 1, Phl p 2, and Phl p 5 [3]. Most subjects had sIgE to Phl p 1 (92%) and sIgE binding to Phl p 5 and Phl p 2 was detected with prevalence of 87% and 59%, respectively. In a study by Marknell et al., 32% of 184 serum samples from grass pollen-sensitized subjects were found to contain sIgE to recombinant Phl p 11 fused with MBP [11]. The sensitization prevalence determined in this study show consistent agreement with the results of the aforementioned study, even though other test methods were used to asses sIgE concentration or patients with clinical symptoms were only included. But in a more extensive study 411 patient sera were evaluated for sIgE by ImmunoCAP to timothy grass pollen components, a higher prevalence rate was found for Phl p 2 (82%), Phl p 5 (92%), Phl p 6 (86%), Phl p 11 (66%), and Phl p 12 (41%) [34]. This increase in prevalence contrasts with Sekerkova et al. study results, who studied patients from the same geographical region - Cuneo (Italy) [3]. The difference could be related to the inclusion criteria. Only samples with sIgE > 0.7 kUa/L to Bermuda grass were included, which were also in high IgE concentration to timothy grass allergens.

Evaluation of the diagnostic performance of timothy grass allergens produced in *N. benthamiana* and *E. coli*

After printing allergens on the microarray and testing with serum samples of known sIgE concentrations, the calibration curves were established for each optimal dilution allergen variant produced in *E. coli* and *N. benthamiana* (Fig. 1).

Subsequently MFI values were converted into arbitrary units (AU) of allergy microarray chip (AMC) (supplementary material 3. Tables) and Spearman correlation coefficient was calculated (Rs). Next, true positives, true negative, false positive and false negative values were determined using reference test cut-off values as a threshold to establish diagnostic sensitivity and specificity. The majority of recombinant allergens tested had specificity < 80% (except for Phl p 11 (*E. coli*) – ISAC ref. and Phl p 12 (*E. coli*) - ALEX ref.) (Table 1). Thus, Youden index was calculated to find optimal cut-off values with the highest sensitivity and specificity values.

For direct comparison of sIgE binding properties on micrroaray Phl p 12 and Phl p 6 were produced in *E. coli* and *N. benthamiana*. Phl p 6 produced in *N. benthamiana* showed overall better sIgE binding performance when compared to *E. coli* produced recombinant allergen (Table 1). Corrected Phl p 6 cut-off values were lower with ISAC and ALEX reference tests (1.45 and 1.52 vs. 2.46 and 1.65 AU, respectively). Hence the sensitivity was also higher in Phl p 6 *N. benthamiana* variant (92% and 85% vs. 81% and 81%). Lower interassay variation was

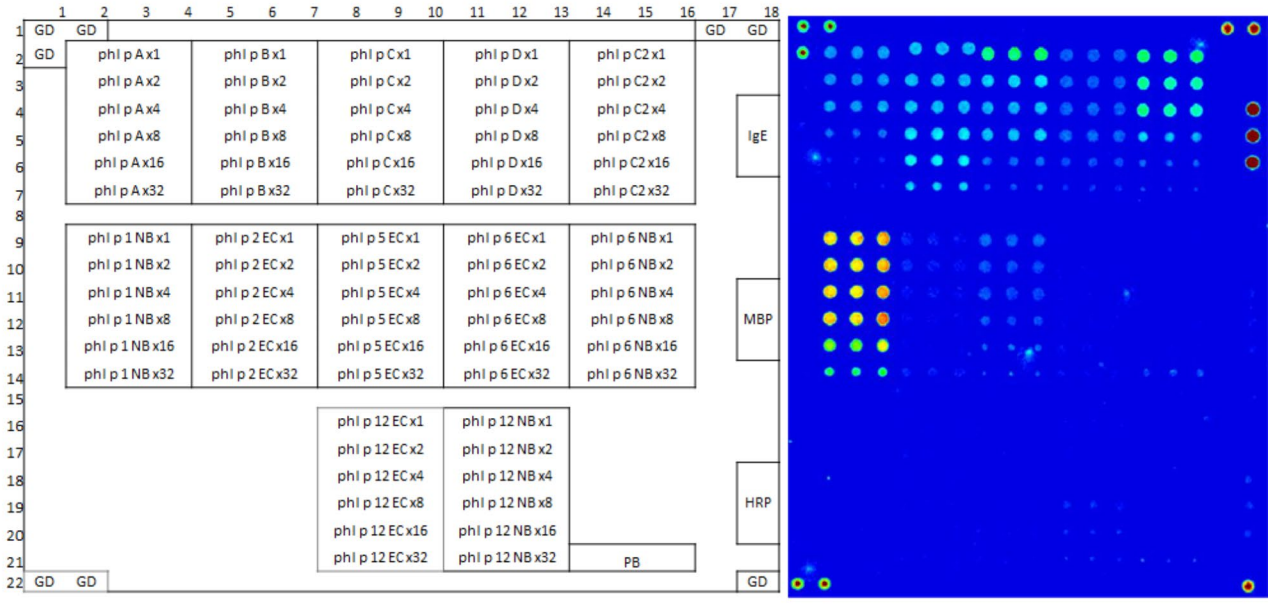


Fig. 1 Allergy microarray chip field print layout and scanned field image in pseudocolors of positive serum sample. GD, guide dot; NT, *Nicotiana benthamiana;* EC, *Escherichia coli*; MBP, maltose binding protein; HRP, horse radish peroxidase; PB, printing buffer; IgE, human IgE antibody; x1-x32, dilution factor; phl p A – D, commercially available timothy extracts from different manufacturers

also observed for Phl p 6 *N. benthamiana* produced protein. On the contrary, Phl p 12 *N. benthamiana* variant had lower diagnostic specificity (76%), despite the fact that according to ALEX test results the Phl p 12 produced in *N. benthamiana* had lower corrected cut-off value 0.61 (AU) versus Phl p 12 *E. coli* produced protein 1.16 (AU). When 0.3 kUa/L cut-off was used then Phl p 12 *E. coli* variant had 90% specificity and Phl p 12 *N. benthamiana* 52% only. In comparison of Phl p 12, the *E. coli* variant had lower inter - assay CV (38%) than *N. benthamiana* variant (53%).

The findings indicate inconsistency of superior quantitative diagnostic performance when directly comparing Phl p 6, Phl p 12 *E. coli* and *N. benthamiana* variants. Similar IgE binding properties were observed aligning with data from other studies. Breiteneder and his collegues using quantitative immunoblotting methods showed that non-purified Bet v 1 overexpressed in *N. benthamiana* had the same immunogenicity as purified Bet v 1 produced in *E. coli* or natural Bet v 1 [35].In another study by Krebitz and collegues reported that sIgE could bind to recombinant Mal d 2 produced in *N. benthamiana*. They used an apple extract and recombinant Mal d 2 to test the sera of apple-allergic patients by immunoblotting. They also showed that recombinant Mal d 2 could block IgE binding to the natural 31 kDa allergen in apple extract, suggesting that recombinant Mal d 2 had the same IgE epitopes as natural Mal d 2 and was immunologically equivalent [5]. In a study led by Üzülmez [36] recombinant Ara h 2 allergens were produced in *N. benthamiana* and *E. coli* and compared to the natural Ara h 2, which outperformed both recombinant proteins in ELISA IgE-binding and activation of basophils via IgE cross-linking. Interestingly, the median-bound IgE value of the plant-made Ara h 2 did not differ significantly from the recombinant Ara h 2 from *E. coli*. Marconi et al. produced Der p 1 in *N. benthamiana* and tested the IgE binding inhibition with human sera and showed a high IgE binding inhibition to the homologous Der p 1 and Der f 1 allergens on the ISAC, suggesting that the recombinant Der p 1 had all the IgE binding epitopes [37]. Yamada et al. [38] compared Bet v 1 produced in *N. benthamiana* and bacteria *B. brevis* sIgE binding capacity using quantitative ELISA method. Both recombinant allergens were found to have comparable binding properties to the IgE of allergic patients.

Overall, both *E. coli* and *N. benthamiana* Phl p 6 and Phl p 12 variants show sIgE binding in a very high positive or high positive correlation (Rs) with the reference tests (supplementary material 1. Figure). Only Phl p 12 *N. benthamiana* with ISAC test showed moderate positive correlation, while *E. coli* variant showed a negligible Spearman correlation that was not even statistically significant to consider. This low correlation could be explained by high reference assay variation when positive serum samples with similar but low sIgE concentration were used (supplementary material 3. Tables).

MBP tag influence on IgE binding properties

For investigation of non-specific IgE binding to MBP tag fused to Phl p 2, Phl p 5 and Phl p 11 allergens, a recombinant MBP was also printed on AMC as control (Fig. 1).

Table 1 Correlation, sensitivity, and specificity of the microarray test based on Timothy Allergens expressed in E. Coli and N. Benthamiana, as determined by the reference tests ALEX and ISAC

Allergen	Origin	Reference test	Rs (CI – 95%)	Sensitivity, %	Specificity %	Cut-off corrected to max Youden index	Sensiti-vity % when cut-off corrected	Specificity % when cut-off corrected	CV %
Phl p 1	*N. benthamiana*	ALEX	0.812* (0.653–0.902)	100	31	1.27	97	94	27
		ISAC	0.945* (0.895–0.971)	100	NA	NA	NA	NA	
Phl p 2	*E. coli*	ALEX	0.810* (0.597–0.917)	100	19	4.10	96	100	41
		ISAC	0.977* (0.948–0.990)	100	73	0.68	92	100	
Phl p 5	*E. coli*	ALEX	0.891* (0.802–0.951)	100	48	2.02	97	100	17
		ISAC	0.853* (0.707–0.930)	100	33	2.41	93	100	
Phl p 6	*E. coli*	ALEX	0.811* (0.615–0.912)	100	21	1.65	81	100	42
		ISAC	0.946* (0.8801–0.9766)	96	23	2.46	81	100	
	N. benthamiana	ALEX	0.816* (0.625–0.915)	100	50	1.52	85	100	34
		ISAC	0.947* (0.8820–0.9770)	96	17	1.45	92	100	
Phl p 11	*E. coli*	ISAC	0.944* (0.801–0.985)	100	95	0.93	100	100	49
Phl p 12	*E. coli*	ALEX	1.00 $p=0.0167$	100	90	1.16	100	100	38
		ISAC	0.385 (-0.229–0.779) $p=0.1942$	100	0	0.96	92	94	
	N. benthamiana	ALEX	1.00 $p=0.0167$	100	52	0.61	100	76	53
		ISAC	0.616 (0.0806–0.876) $p=0.0277$	100	5	0.80	85	68	

NA, not applicable; Rs, Spearman correlation coefficient; CV, coefficient of variation; CI, confidence interval;

* $p<0.0001$.

As analyzed by SDS-PAGE, all tested allergens were obtained in electrophoretically pure states, but immunoblot analysis showed that Phl p 5 and Phl p 11 were partially degraded (Fig. 2).

The degradation of MBP fused Phl p 5 resulted in a subunit of about 32 kDa, which matched the molecular weight of Phl p 5. On AMC with all tested positive and negative serum samples, no high MFI values for MBP as control spot were observed, indicating the absence of sIgE against MBP in serum samples. Similar finding was found in dot blot experiments when Cyp c 2 MBP fused allergen reacted specifically with 3 positive patient samples, but not MBP [14].

In a study by Marknell DeWitt et al., when a larger serum sample size ($n=188$) was tested, a small proportion (7%) of the samples positive for MBP-fused Phl p 11 also displayed reactivity to MBP alone. This indicates that the MBP fusion protein may introduce some false positive results in the sIgE assay [11]. Similar observations were made in another study, where MBP-fused Ara h 2 exhibited a 90% reduction in sIgE binding compared to the native allergen in a subset of samples ($n=15$). However, in another subset ($n=14$), IgE binding to MBP-fused and native allergens showed a strong correlation [39]. These findings suggest that while MBP fusion enhances protein solubility, it may also introduce variability in IgE reactivity. Therefore, careful consideration must be given when designing MBP-fused allergens to minimize the risk of false results in diagnostic applications.

The scanner signal data of different allergen variants (Phl p 6 and Phl p 12) showed that the MBP-fused *E. coli* variants had much higher average MFI values than the *N. Benthamiana* variants (data not shown). This is consistent with the findings of other study [13] where ELISA results did not show high differences in MFI between the *E. coli* produced Pen m 4 and MBP-fused

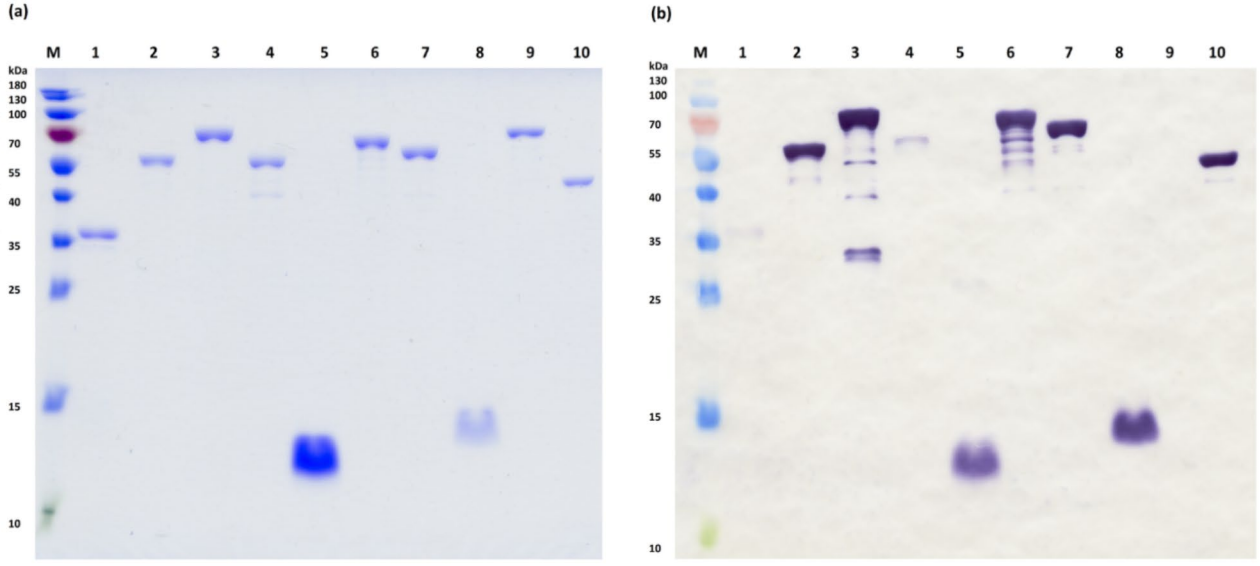


Fig. 2 Analysis of purified Phl p allergens by **(a)** SDS-PAGE and **(b)** Immunoblot. Lanes: [M], PageRuler™ Prestained Protein Ladder (ThermoFisher Scientific, Lithuania); [1], *N. benthamiana* produced Phl p 1; [2], *E. coli* produced Phl p 2 fused with MBP; [3], *E. coli* produced Phl p 5 fused with MBP; [4], *E. coli* produced Phl p 6 fused with MBP; [5], *N. benthamiana* produced Phl p 6; [6], *E. coli* produced Phl p 11 fused with MBP; [7], *E. coli* produced Phl p 12 fused with MBP; [8], *N. benthamiana* produced Phl p 12; [9] BSA protein, acts as a negative control for Immunoblot; [10] 6xHis tag MBP protein acts as a positive control for Immunoblot with Tetra His antibodies (1:1000 in PBS-T, QIAGEN, Cat. No. 34670, Germany). The raw images can be found in the Supplementary material 2. Raw images

Pen m 4 variants, but MBP-Pen m 4 showed significantly higher MFI levels in the protein microarray. Since MBP part of fused allergen might participate in the covalent attachment to the surface of microarray, it can increase the number of allergen epitopes available for antibody binding. In contrast, in ELISA, where allergen is used excessively, the effect of epitope hindrance could be less pronounced.

Determination of inter-assay variation

To determine the inter-assay CV of recombinant timothy grass allergens, positive serum samples were re-tested after 7 weeks. The glass slides were stored at +4 °C, and serum samples were frozen and thawed again before re-testing. Overall inter-assay CV values varied between recombinant allergens from 17% to 53% (Table 1.) When compared to other microarray study results of assay variability [40, 41], only Phl p 5 showed comparably low CV values, while the remaining allergens CV values were above 25%. In microarray study led by Jahn-Schmid [42] the batch-to-batch variation CV values calculated for the Phl p 1, Phl p 2, Phl p 5 and Phl p 6 were 25, 27, 36, and 47% respectively. Our finding reveals that high inter-assay variation exept to to Phl p 5 (CV = 17%) values might be associated with immobilized protein instability during prolonged storage, since MFI values after re-test were found to be lower than on the first testing day.

Conclusions

Allergen components are emerging as a superior alternative to traditional extract-based allergens in allergy diagnostics. They can improve test resolution by differentiating between molecular cross-reactivity and true co-sensitization, and they can also provide a more accurate prediction of the potential type of clinical reaction. Our study was designed to assess and compare diagnostic performance of differently produced recombinant timothy grass allergens on a microarray. The patient sera samples used in this study had similar sensitization profiles as in other studies, despite regional differences.

Of all tested proteins, only MBP-fused Phl p 11 had high specificity and sensitivity comparable to commercial multiplex tests. Phl p 6 and Phl p 12 allergens produced in *E. coli* or *N. benthamiana* performed very similarly in microarray experiments, which correlates with previous findings. In contrast to some other studies, MBP as a fusion protein did not show any negative influence on sIgE binding to timothy allergens. Fused allergens showed even higher sIgE binding in microarray experiments, suggesting that MBP-fused allergens can be advantageous in microarray applications. Because of the reasons stated above, we recommend the use of *E. coli* produced MBP fused recombinant Phl p 6, and Phl p 12 allergens, as recombinant allergen production *E. coli* is simpler and more scalable compared to *N. benthamiana*.

Our results also showed high intraassay variation, probably related to protein instability. Thus, improvements in

allergen formulation, printing process and microarray storage conditions should be studied further.

Supplementary Information

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Supplementary Material 1. Figure

Supplementary Material 2. Raw images

Supplementary Material 3. Tables

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
The manuscript does contain studies with human serum samples. Lithuanian Bioethics Committee for Biomedical Research approved the study protocol (Nr. 158200–17–926–430). We obtained informed consent from all participants before conducting the study. The consent was written and signed by the participants, and we kept a copy of the consent forms for our records. For participants who were minors, we also obtained written consent from their parents or guardians. We followed the ethical guidelines of our institution and the relevant regulations of our country regarding informed consent.

Consent for publication
Not applicable.

Competing interests
LS, LT, LG reports a relationship with Imunodiagnostika that includes: employment. Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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