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# Allergen content in German cockroach extracts and sensitization profiles to a new expanded set of cockroach allergens determine *in vitro* extract potency for IgE reactivity

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## Methods

### *Measurement of Bla g 1, Bla g 2 and Bla g 5 in cockroach extracts by ELISA*

Levels of Bla g 1, Bla g 2 and Bla g 5 in cockroach extracts were measured by ELISA. Purified recombinant Bla g 1, purified natural Bla g 2 and purified recombinant Bla g 5, all prepared in 1% BSA/50% glycerol/PBS, pH 7.4, were used as standards. The concentration of the three purified allergen standards was determined by amino acid analysis. The antibody pairs used in each assay are specified in parenthesis: Bla g 1 (10A6/pAb), Bla g 2 (7C11/pAb) and Bla g 5 (17B12/pAb). Each extract was analyzed at two starting concentrations (1:10 and 1:1,000) with 11 doubling dilutions across the plate and tested in triplicate for each dilution. The pAb were detected with peroxidase-labelled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Assay development was performed as for IgE antibody inhibition assays.

### *Endotoxin measurement*

Each cockroach extract was analyzed for endotoxin content using the chromogenic *Limulus* Amoebocyte Lysate (LAL) assay (Lonza, Walkersville, MD). The extract was analyzed at a starting concentration of 1:100 with three 1:5 dilutions up to 1:12,500 and results were reported in Endotoxin Units (EU) per milliliter of extract.

### *Expression, purification and quantification of eight recombinant cockroach allergens*

German cockroach allergens Bla g 2 (GenBank accession code U28863), Bla g 4 (U40767), Bla g 6 (DQ279092) were constitutively expressed in *Pichia pastoris* using pGAPZα vectors, while Bla g 1 (AF072219), Bla g 9 (DQ358231), Bla g 11 (DQ355516) were expressed using pPICZ/pPICZα vectors by methanol induction for 48-96 hours. Per a 7 (isoform Per a 7.0102;

AF106961) was expressed by methanol induction using the *Pichia* pPIC9 vector. Per a 7.0102 is highly cross-reactive and shares 98.6% identity with Bla g 7.0101. Bla g 5 (U92412) was expressed in *Escherichia coli* using the pET-21a vector.

Bla g 1, Bla g 2 and Per a 7 were purified by specific-antibody affinity chromatography. Bla g 4 was purified by phenol-sepharose chromatography, Bla g 5 by glutathione *S*-transferase affinity chromatography, and Bla g 6 by ion exchange and size exclusion chromatography. Bla g 9 and Bla g 11 were purified by metal affinity chromatography. Bla g 1, Per a 7, Bla g 9 and Bla g 11 were quantified by Advanced Protein Assay (Cytoskeleton Inc., Denver, CO) while Bla g 2, Bla g 4, Bla g 5, and Bla g 6 by $OD_{280}$.

***Biotinylation and optimization of biotinylation***

EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, Rockford, IL) was added to 2 mg of each allergen at a 10-20-fold molar excess, depending on the number of lysine residues in the sequence, and incubated for 30 minutes. The biotinylated mix was put over a pre-washed Zeba Desalt Spin Column (Thermo Scientific, Rockford, IL) 2 times and the concentration was determined after biotinylation by APA.

The quantification of biotinylation was carried out by using a Quant Tag™ Biotin Kit (Vector Laboratories, Burlingame, CA). Samples were tested in triplicate against a known biotin standard curve to determine the number of biotins per allergen molecule. Optimal number of biotins per molecule was considered between 2 and 6.

***Optimization of biotinylated allergen loaded to the streptavidin ImmunoCAP***

Streptavidin ImmunoCAPs (Thermo Fisher Scientific, Portage, MI) were loaded and incubated on a Phadia 100, with the biotinylated allergen at the following amounts: 0.5, 1, 2, 5, 10 µg/CAP. Two different human plasma samples from individuals allergic to the allergen (that had been originally tested for IgE binding to 3 µg/CAP) were selected for optimization experiments. IgE binding to the allergen-loaded CAPs by the two selected plasma was measured in a Phadia 250 following manufacturer's instructions (Thermo Fisher Scientific, Portage, MI). Results were plotted to select optimal amount of biotinylated allergen to be loaded to the streptavidin ImmunoCAPs.

***Measurement of IgE antibody levels by ImmunoCAP***

Biotinylated allergen was loaded and incubated on streptavidin ImmunoCAPs using the Phadia 100. The ImmunoCAPs were transferred to the Phadia 250, where measurements of IgE antibody binding were performed according to manufacturer's instructions. Cockroach-specific IgE antibody binding was measured using commercially available CAPs loaded with cockroach extract (i6 ImmunoCAPs supplied by Thermo Fisher Scientific). Most subjects (except 3) did not have IgE antibodies against at least one of the 8 allergens. These negative IgE values served as negative controls and indicated that positive values were allergen-specific. Also, sera from non-cockroach allergic patients (n = 10) were used as negative controls. These sera were negative at a cut-off of 0.1 $kU_A/L$ in in-house streptavidin ImmunoCAPs either not loaded with allergen or loaded with each of 7 cockroach allergens (data not shown). Regardless, a conservative cut-off of 0.35 $kU_A/L$ was chosen to make sure that IgE prevalences would not be overestimated due to low values between 0.1 and 0.35 $kU_A/L$. In addition, and to assess possible non-specific IgE binding,

all plasma samples were run in streptavidin-CAPs not loaded with allergen. CCD binding to the allergens was not expected for most allergens since only three had N-glycosylation sites (two in Bla g 2 and one in Bla g 4 and Bla g 11). For the three plasma samples that reacted to all 8 allergens, a test was run with a CCD inhibitor for these three allergens, to assess possible IgE binding to the carbohydrates (as explained below).

IgE binding to CCD present in allergens that contain N-glycosylation was assessed by adding a CCD-inhibitor to the plasma before measuring IgE binding to rBla g 2 and rBla g 4 (rBla g 1 was used as a negative control because it lacks N-glycosylation sites). The lyophilized RIDA CCD-Inhibitor (R-Biopharm AG, Darmstadt, Germany) was dissolved in sterile $H_2O$, with vortexing. The CCD-Inhibitor (or sterile $H_2O$ for corresponding sample without inhibitor) was added at a dilution of 1:41 to sample plasma and incubated on an orbital shaker for one hour at room temperature. Samples were run on the Phadia 250 immediately after incubation.

***Specificity of the allergen-specific IgE measurements by ImmunoCAP***

The IgE measurements for the component analysis were allergen specific. This was proven by: 1) all plasma had IgE antibody levels to streptavidin-CAPs (not loaded with allergen) that were under the cut-off of 0.35 $kU_A/L$ (except one with a low value of 0.59 $kU_A/L$ that was used to correct the allergen-specific levels (by subtracting 0.24 $kU_A/L$, the difference between 0.59 and the cut-off); 2) most plasma did not bind one or more of the eight allergens (except three plasma that bound the 8 allergens), and these measurements acted as negative controls, and 3) the only three plasma with positive IgE values to all eight allergens tested, showed no difference in IgE

levels in presence versus absence of CCD inhibitor for two allergens with N-glycosylation (Bla g 2 and Bla g 4) and without as a control (Bla g 1).

175 **Results**

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177 **Table E1.** Cockroach-specific IgE, skin prick test wheal size, age and gender of the study cohort
178 of 23 individuals sensitized to cockroach.

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**Information on cockroach allergic donors**

| Subject # | Donor ID | CR-specific IgE ($kU_A/L$) | SPT Wheal size (mm) | Age | Gender |
|---|---|---|---|---|---|
| 1 | 1441 | 0.91 | 0 | 47 | M |
| 2 | 1439 | 0.94 | 8 | 32 | M |
| 3 | 2196 | 1.23 | n.d. | 53 | M |
| 4 | 1367 | 1.27 | 6 | 37 | F |
| 5 | 1006 | 1.32 | 0 | 44 | M |
| 6 | 1365 | 2.01 | 4.5 | 49 | F |
| 7 | 1665 | 2.24 | n.d. | 26 | F |
| 8 | 2083 | 3.41 | n.d. | 23 | F |
| 9 | 1231 | 4.47 | 3.5 | 23 | F |
| 10 | 1257 | 4.78 | 6 | 37 | F |
| 11 | 1864 | 4.82 | n.d. | 37 | F |
| 12 | 1406 | 5.30 | 4.5 | 41 | F |
| 13 | 1175 | 7.27 | 3 | 43 | F |
| 14 | 1437 | 8.32 | 9 | 38 | F |
| 15 | 2210 | 10.13 | n.d. | 28 | F |
| 16 | 1398 | 10.50 | 8.5 | 30 | F |
| 17 | 1229 | 12.20 | 9 | 49 | M |
| 18 | 1446 | 17.30 | 7.5 | 50 | M |
| 19 | 1425 | 36.00 | 7 | 39 | F |
| 20 | 1424 | 45.20 | 10 | 30 | F |
| 21 | 1228 | 56.50 | 7 | 54 | F |
| 22 | 1277 | 66.20 | 10 | 53 | M |
| 23 | 1445 | 76.20 | 9 | 32 | F |
| | Average | 16.46 | | 38.9 | 69.6% F |
| | Std deviation | 22.76 | | 9.8 | 30.4% M |

* n.d. not determined

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**Table E2**. Correlations of extract potencies (IC30) for IgE reactivity with 13 extracts for 5 subjects. Single or double underlining of the donor ID denote the two groups of subjects identified. Within each group, the correlations between pairs of subjects were significant (in bold; $p < 0.001$).

| | **1445** | **1277** | **1424** | **1425** | **1864** |
|---|---|---|---|---|---|
| **1445** | 1 | | | | |
| **1277** | **0.947** | 1 | | | |
| **1424** | 0.550 | 0.502 | 1 | | |
| **1425** | 0.447 | 0.436 | **0.940** | 1 | |
| **1864** | 0.426 | 0.372 | **0.941** | **0.989** | 1 |

**Table E3.** Correlations of eight allergen-specific IgE for 5 subjects. The best correlation was between subjects 1445 and 1277 ($r = 0.693$, $p = 0.057$).

| | **1445** | **1277** | **1424** | **1425** | **1864** |
|---|---|---|---|---|---|
| **1445** | 1 | | | | |
| **1277** | 0.693 | 1 | | | |
| **1424** | 0.249 | 0.170 | 1 | | |
| **1425** | 0.346 | 0.325 | 0.499 | 1 | |
| **1864** | 0.136 | 0.010 | 0.559 | 0.331 | 1 |

**Table E4.** Correlations between extract potencies for IgE reactivity and allergen content of the twelve extracts for five cockroach allergic patients.

| | **Correlations between extract potencies (IC30) and extract allergen content (µg/mL)** | | | | | | **Allergen-specific IgE** ($kU_A/L$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Bla g 1 + Bla g 2 + Bla g 5 | | Bla g 1 | | Bla g 2 | | | | |
| **Patients** | r | p | r | p | r | p | Bla g 1 | Bla g 2 | Bla g 5 |
| 1445 | 0.779 | *0.0028 | 0.871 | *0.0002 | 0.469 | 0.1245 | 14.06 | 1.98 | 8.45 |
| 1277 | 0.773 | *0.0032 | 0.838 | *0.0007 | 0.527 | 0.0784 | 7.56 | 5.82 | 0.45 |
| 1424 | 0.026 | 0.9358 | 0.123 | 0.7035 | 0.193 | 0.5481 | 1.34 | 8.83 | 9.81 |
| 1425 | 0.306 | 0.3337 | 0.265 | 0.4052 | 0.357 | 0.2540 | < 0.35 | 0.7 | 8.14 |
| 1864 | 0.375 | 0.2302 | 0.476 | 0.1181 | 0.098 | 0.7613 | < 0.35 | 2.03 | < 0.35 |

Pearson's correlation coefficient r

* $p < 0.05$ indicates significance

**Table E5.** Correlations, for each extract, between German cockroach extract potencies and the sum of allergen-specific IgEs of five subjects analyzed.

| | **Correlations between extract potencies (IC30) and** | | | | **Three allergens/ protein concentr. per extract (µg/mg)** |
|---|---|---|---|---|---|
| | **Sum IgE to 3 allergens* ($kU_A/L$)** | | **Sum IgE to 8 allergens ($kU_A/L$)** | | |
| Extract | **r** | **p** | **r** | **p** | |
| 1 | 0.395 | 0.5108 | 0.943 | 0.0162 | 10.32 |
| 2 | 0.024 | 0.9678 | 0.382 | 0.5254 | 4.20 |
| 3 | 0.621 | 0.2635 | 0.896 | 0.0397 | 4.87 |
| 4 | 0.139 | 0.8241 | 0.608 | 0.2764 | 4.56 |
| 5 | 0.696 | 0.1921 | 0.977 | 0.0041 | 12.33 |
| 6 | 0.407 | 0.4969 | 0.765 | 0.1319 | 4.77 |
| 7 | 0.452 | 0.4449 | 0.972 | 0.0056 | 10.32 |
| 8 | 0.037 | 0.9521 | 0.746 | 0.0466 | 8.16 |
| 9 | n/a | n/a | n/a | n/a | 10.27 |
| 10 | 0.275 | 0.6540 | 0.914 | 0.0297 | 26.02 |
| 11 | 0.552 | 0.3343 | 0.945 | 0.0154 | 29.33 |
| 12 | 0.553 | 0.3341 | 0.956 | 0.0109 | 53.86 |
| 13 | n/a | n/a | n/a | n/a | 0 |

*Bla g 1, Bla g 2 and Bla g 5

Pearson's correlation coefficient (r)

$p < 0.05$ indicates significance (data associated with a significant correlation are underlined)

n/a: Not applicable (#9 is the reference extract and #13 is the negative control)

**Figure legends**

**Figure E1.** Proportion of allergen-specific IgE versus the sum of eight allergen-specific IgE in the cockroach allergic subjects (n = 23). This figure represents a normalization to percentages of data from Figure 2, including data below the 0.35 $kU_A/L$ threshold (as 0.35 values).

**Figure E2.** Inhibition assays to determine the *in vitro* potencies for IgE reactivity of extracts in five cockroach allergic subjects. Results are from four representative subjects out of five analyzed. Plots show means with standard deviations of duplicates. The reference curves for each of the three plates used in the experiment are #9-1, #9-2 and #9-3.

**Figure E1.**

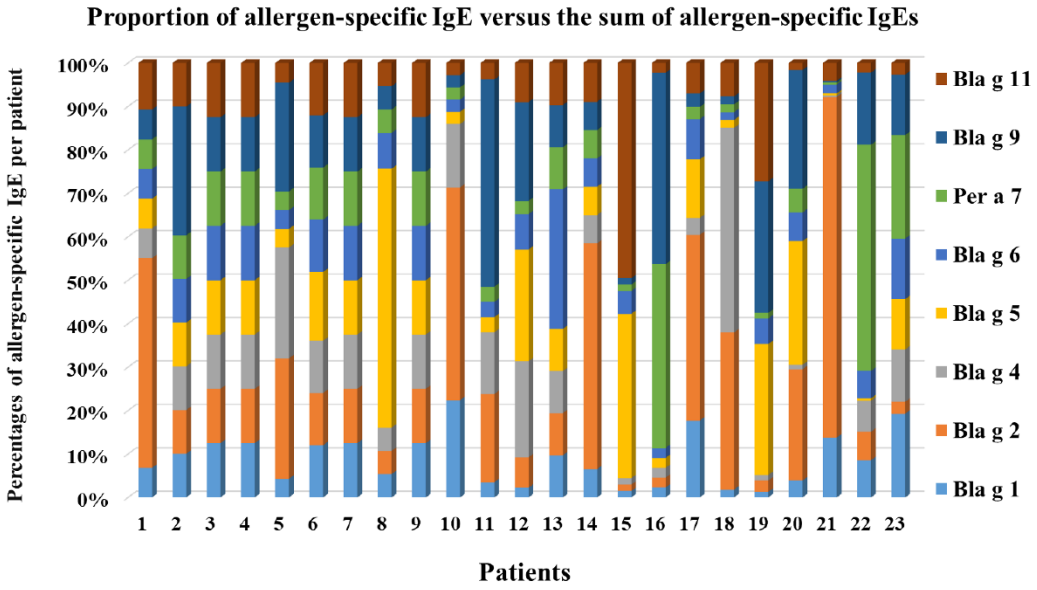
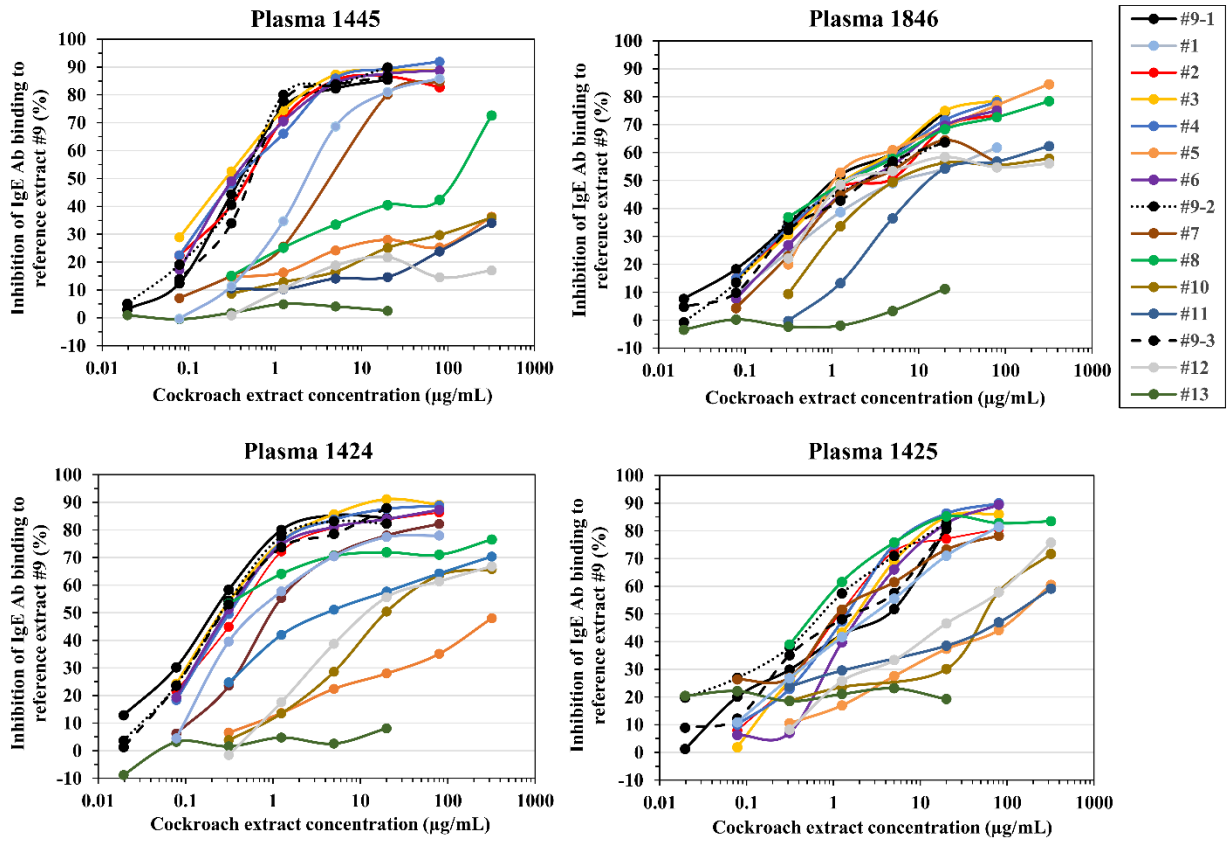

Proportion of allergen-specific IgE versus the sum of allergen-specific IgEs
Percentages of allergen-specific IgE per patient
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Patients
Bla g 11
Bla g 9
Per a 7
Bla g 6
Bla g 5
Bla g 4
Bla g 2
Bla g 1

**Figure E2.**


Plasma 1445
Plasma 1846
Plasma 1424
Plasma 1425
Inhibition of IgE Ab binding to reference extract #9 (%)
Cockroach extract concentration (µg/mL)
#9-1
#1
#2
#3
#4
#5
#6
#9-2
#7
#8
#10
#11
#9-3
#12
#13