frontiers in Allergy

ORIGINAL RESEARCH
published: 29 June 2021
doi: 10.3389/falgy.2021.691627

Component-Resolved Diagnosis of American Cockroach (*Periplaneta americana*) Allergy in Patients From Different Geographical Areas

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Specialty section:
This article was submitted to Allergens, a section of the journal Frontiers in Allergy

Received: 06 April 2021
Accepted: 03 June 2021
Published: 29 June 2021

Citation:
Wangorsch A, Jamin A, Eichhorn S, Pablos I, Sharma S, Schweidler B, Kastner B, Wildner S, Saloga J, Führer F, Reyna Orozco RR, Sherkat R, Sadeghi S, Teifoori F, Park J-W, Briza P, Vieths S, Ferreira F, Arora N, Lidholm J, Gadermaier G and Scheurer S (2021) Component-Resolved Diagnosis of American Cockroach (Periplaneta americana) Allergy in Patients From Different Geographical Areas. Front. Allergy 2:691627. doi: 10.3389/falgy.2021.691627

Background: Manifestation of respiratory allergy to American cockroach (*Periplaneta americana*) is prominent in the subtropical and tropical areas. However, co-existing perennial indoor inhalant allergies frequently compromise clinical diagnosis of cockroach allergy, and the analysis of sensitization pattern is limited by the lack of *Periplaneta* allergens widely available for component-resolved diagnostics (CRD).

Objective: To evaluate a collection of previously described recombinant *Periplaneta* allergens for CRD in cockroach allergy.

Methods: A panel of nine recombinant *Periplaneta* allergens (Per a 1–5, 7–10) was generated, purified, and subjected to physicochemical characterization by applying circular dichroism (CD) spectroscopy, dynamic light scattering (DLS), amino acid (AA) analysis, and mass spectrometry (MS). Patients ($n = 117$) from India, Korea, Venezuela, and Iran, reporting perennial respiratory indoor allergies with IgE sensitization to cockroach (*P. americana and/or Blattella germanica*), were included. The sensitization profile was monitored by the experimental ImmunoCAP testing.

Results: ImmunoCAP testing confirmed IgE sensitization to *Periplaneta* and/or *Blattella* extract in 98 of 117 patients ($r = 0.95$). Five out of 117 patients were sensitized to only one of the two cockroach species. Within the whole study group, the prevalence of sensitization to individual allergens varied from 4% (Per a 2) to 50% (Per a 9), with the highest IgE values to Per a 9. Patients from four countries displayed different sensitization profiles at which Per a 3 and Per a 9 were identified as major allergens in India and Korea. *Periplaneta*-derived lipocalin and myosin light chain were characterized as new minor allergens, designated as Per a 4 and Per a 8. *Periplaneta* extract showed higher

diagnostic sensitivity than all individual components combined, suggesting the existence of allergens yet to be discovered.

Conclusion: Utilization of a panel of purified *Periplaneta* allergens revealed highly heterogeneous sensitization patterns and allowed the classification of lipocalin and myosin light chain from *Periplaneta* as new minor allergens.

Keywords: cockroach allergy, American cockroach, *Blattella germanica*, component-resolved diagnosis, *Periplaneta americana* (Insecta)

INTRODUCTION

The most common indoor aeroallergens are house dust and storage mites, pet dander, mold, and cockroaches (1). German cockroach (*Blattella germanica*), American cockroach (*Periplaneta americana*), Oriental cockroach (*Blatta orientalis*), brown-banded cockroach (*Supella longipalpa*), and smoky brown cockroach (*Periplaneta fuliginosa*) have been reported to cause allergic asthma morbidity worldwide (2). Cockroach allergy is predominantly caused by *Blattella* and *Periplaneta* in temperate and (sub)tropical areas and is a global health problem due to the increasing infestation of cockroaches in human housing environments (3). Cockroach allergens are present in saliva, fecal particles, spermatophores, shredded skin, and desiccated remains of insect bodies (2). In studies including children and adults, presented to hospital, the prevalence of cockroach allergy ranges from 17 to 41% in the United States, and 60–80% of inner-city children with asthma were sensitized to cockroach. It has been suggested that exposure to cockroach allergens appears to have a greater effect on asthma morbidity than dust mite or pet allergens, in particular among inner-city children (2).

Allergic sensitization to cockroach is frequently investigated by skin testing using crude extracts and by the measurement of specific IgE to cockroach allergens (2). However, the usage of allergen extracts possesses some limitations. Commercial or self-prepared allergen extracts at most are standardized based on in-house assays, causing the lack of comparability between different manufacturers. Moreover, the lack of immune-dominant allergen(s) and the complex patterns of IgE responses to cockroach extracts have made it difficult to produce standardized cockroach allergen extracts (4). Therefore, one approach to overcome these drawbacks is the utilization of purified allergens for component-resolved diagnostics (CRD).

Whereas, allergic sensitization to *B. germanica* allergens has been investigated in detail, data referring to *Periplaneta* allergens are less available. However, the tested cockroach species frequently is not indicated in the respective reports. Of note, species-specific sensitization to *Periplaneta* and *Blattella* has been reported, and in one study, only 68% of *Blattella*-reactive patients showed sensitization to *Periplaneta* (5). *Vice versa*, in China, 25.7% of allergic patients were sensitized to *Periplaneta* allergens, whereas only 18.7% were sensitized to *Blattella* allergens (6), indicating allergenic differences between these cockroach species or different diagnostic sensitivity of the extracts used. Although homologous allergens have been described for *Periplaneta* and *Blattella*, the presence of different allergenic components between the species needs to be considered. So far, among 13 suggested *Periplaneta* allergens, 11 have been officially recognized by the WHO/IUIS Allergen Nomenclature Sub-Committee (7, 8). Per a 1 (25–45 kDa, midgut microvilli-like protein) (9–11), Per a 2 (42 kDa, aspartic protease-like protein) (12), Per a 3 (46–79 kDa, homologue of arylphorin and insect hemocyanin) (13, 14), Per a 5 (23 kDa, glutathione S-transferase) (15–17), Per a 6 (18 kDa, troponin C) (18), Per a 7 (33 kDa, tropomyosin) (19, 20), Per a 9 (43 kDa, arginine kinase) (21, 22), Per a 10 (28 kDa, serine protease) (23), Per a 11 (55 kDa, α-amylase) (24), Per a 12 (45 kDa, chitinase) (24), and Per a 13 (36 kDa, glyceraldehyde-3-phosphate dehydrogenase) (24). Although lipocalin (Acc.No. AY792948) (25, 26) and myosin light chain (Acc.No. JQ279816, unpublished) have been suggested as potential *Periplaneta* allergens and have been proposed as Per a 4 and Per a 8, they are less well-characterized.

So far, studies addressing the sensitizing capacity of *Periplaneta* allergens were mainly performed with selected allergens rather than by CRD using the panel of *Periplaneta* allergens. Of note, data on the prevalence of IgE binding to target allergens are highly variable between different studies (7). An ELISA-based CRD study conducted in Taiwan, enrolling *Blattella*-sensitized patients with persistent asthma and including recombinant Per a 1 through Per a 7 and Per a 9, revealed a reactivity with Per a 2 and Per a 9 to be associated with severe asthma and allergic rhinitis, respectively (12). Taken together, the reports on the contribution of individual *Periplaneta* allergens in cockroach allergy are not consistent. It is worth noting that study results on the clinical significance of individual *Periplaneta* allergens may vary substantially depending on the quality of purified allergens used for antibody detection, patient inclusion criteria, co-exposure to cross-reactive allergens from other sources, and the geographical area where the study is conducted.

The present study aimed to investigate the molecular sensitization profile in a substantial number of cockroach-sensitized patients recruited in four different countries, all with perennial respiratory indoor allergy. For the purpose of CRD, a set of nine recombinant *Periplaneta* allergens were produced and characterized by uniform methods.

MATERIALS AND METHODS

Patients and Patient's Sera

For CRD, patients ($n = 117$) reporting perennial respiratory indoor allergy with confirmed IgE sensitization to *P. americana* and/or *B. germanica* were included in the study. Patients'

sensitization to cockroach was monitored by IgE-ELISA (India, $n = 35$) and IgE-immunoblotting (Iran, $n = 30$) using the self-prepared *Periplaneta* extract, by SPT using *Blattella* extract (ALK, Round Rock, TX, United States) (Venezuela, $n = 25$), and *Blattella* extract ImmunoCAP (i6, Thermo Fisher Scientific, Uppsala, Sweden) (Korea, $n = 27$), respectively. Screening of *Periplaneta* allergen expression and purification was done using sera from cockroach-allergic patients (Germany, $n = 1$; Plasmalab, Everett, WA, United States, $n = 6$). Ethical approval was obtained from IHEC (no. CLP 0019, CSIR-IGIB) and Isfahan University of Medical Sciences and Health Services (no. 295264).

Preparation of *P. americana* Extract

Periplaneta americana extract was produced as described in the **Supplementary Material**. In brief, proteins were extracted from lyophilized powder of defatted whole-body *Periplaneta* cockroaches using phosphate-buffered saline (PBS), and the protein fractions were gained after subsequent centrifugation and filtration steps.

Generation and Physicochemical Characterization of Recombinant *Periplaneta* Allergens

Detailed steps for the preparation of recombinant Per a allergens are depicted in the **Supplementary Material**. For cDNA cloning, published GenBank amino acid (AA) sequences were used as template, and signal peptides were excluded from the sequence. Five out of the nine proteins were produced as non-tagged proteins. Per a 2 and Per a 3 contain a C-terminal His_{tag}, whereas Per a 5 and Per a 10 comprise a N-terminal His_{tag} for purification. All proteins were expressed in *Escherichia coli* and purified by multiple chromatographic steps. Final protein concentration and purity was checked by bicinchoninic acid (BCA) (Sigma-Aldrich, United States), amino acid analysis (AAA), and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Additionally, mass spectrometry (MS) (intact masses and tryptic digestion) analysis was performed to confirm the identity of recombinant proteins. To determine the structural integrity and the aggregation status of the proteins, circular dichroism (CD), and dynamic light scattering (DLS) were carried out. Recombinant expression of individual allergens and analytical methods are described in the **Supplementary Material**.

IgE Immunoblot and CRD

Immunoblotting was performed to (1) demonstrate IgE sensitization of Iranian patients recruited by clinical history (not shown) and (2) to assess the IgE sensitization pattern for selected patients lacking detectable IgE binding to purified *Periplaneta* allergens. Briefly, *P. americana* extract (50 μg/cm) was applied to SDS–PAGE, transferred to nitrocellulose membrane (0.2 μm, Amersham Protean, GE Healthcare, Freiburg, Germany) by semi-dry blotting, and visualized by Ponceau S staining (Sigma-Aldrich, Munich, Germany). After blocking with 0.3% Tween-20 in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.4), the membrane was incubated overnight with patient's serum (500 μl/strip, diluted 1:10 in TBS, 0.05% Tween-20, 0.1% BSA), and bound IgE was detected as described elsewhere (27).

Specific IgE values to *Periplaneta* (i206) and *Blattella* extract (i6) were determined by ImmunoCAP tests (Thermo Fisher Scientific) and to single recombinant *Periplaneta* allergens by experimental ImmunoCAP tests as previously described (28). Assay background of each test was assessed and cutoff level for positivity adapted as required (0.35 kU_A/L for Per a 2, Per a 5, and Per a 10 and 0.10 kU_A/L for all other allergens).

RESULTS

Generation of Recombinant *Periplaneta* Allergens

All recombinant *Periplaneta* allergens were expressed in *E. coli* as non-fusion as well as N- or C-terminal His_{tag} proteins. After multistep chromatography, purified proteins were characterized by uniform physicochemical methods as indicated in **Supplementary Table 1**. Purity and apparent molecular weight of all proteins were assessed by SDS–PAGE performed under reducing conditions (**Figure 1**).

The identity of all nine recombinant *Periplaneta* proteins included in the study was confirmed by MS analysis, by either intact mass analysis or in-solution/in-gel digestion and AA analysis. The identity of Per a 6 could not be confirmed, and it was therefore excluded from the study. Of note, the MS analysis revealed that a high proportion of Per a 8 had a truncation at the N-terminus of the protein, with only 10% being present as the full-length protein (**Supplementary Table 1**). The truncation observed for Per a 8 did not affect the secondary structure of the protein. CD spectroscopy confirmed that most of the allergens show structural integrity or were at least partially folded (**Supplementary Table 1**). Analysis by DLS revealed that some recombinant allergens tended to partially aggregate; however Per a 4, Per a 8, and Per a 9 were determined as mostly monomeric proteins.

IgE Sensitization to *Periplaneta* and *Blattella* Allergens

Periplaneta- and *Blattella*-specific IgE was analyzed by ImmunoCAP testing in all 117 serum samples (**Figure 2**),

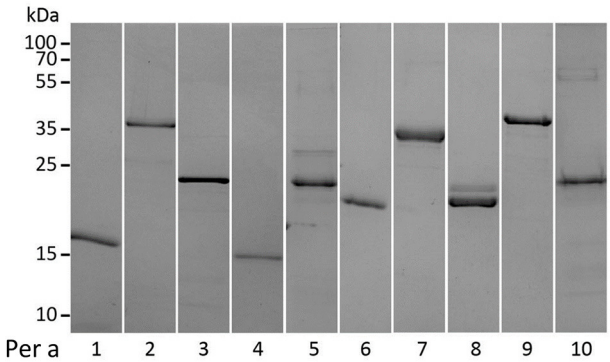

FIGURE 1 | SDS–PAGE and Coomassie staining of recombinant *Periplaneta* allergens, Per a 1 to Per a 10 (1-10), under reducing conditions.

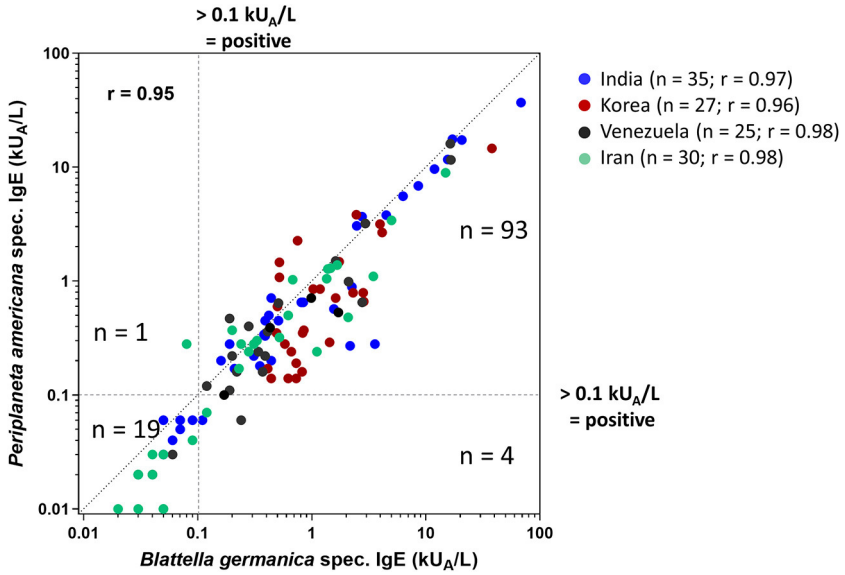

FIGURE 2 | Comparison of *Periplaneta* (i206)- and *Blattella* (i6)-specific IgE values in 117 patients (India, $n = 35$; Korea, $n = 27$; Venezuela, $n = 25$; Iran, $n = 30$) reporting cockroach allergy by ImmunoCAP testing (cutoff 0.1 kU$_A$/L); *r*-values are indicated.

showing an overall interspecies correlation of $r = 0.95$ and $r = 0.96$–0.98 for individual patient collectives. *In vitro* IgE reactivity to *Blattella* and *Periplaneta* was confirmed in 83% (97/117) and 81% (95/117) of the patients, respectively. Four subjects were sensitized to *Blattella* but not to *Periplaneta*, and one was sensitized to *Periplaneta* but not to *Blattella*. In total, 98 of 117 patients reacted to one or both of the cockroach species (**Figure 2**). Although patients were preselected by sensitization to cockroach by ELISA, SPT, and immunoblotting, 19/117 sera (comprising 6/35 from India, 3/25 from Venezuela, 10/30 from Iran) were tested negative to both species by ImmunoCAP.

Component-resolved Diagnosis of *Periplaneta* Allergy by ImmunoCAP Testing

In total, nine recombinant *Periplaneta* allergens (Per a 1 to Per a 10, except Per a 6) were included for CRD by experimental ImmunoCAP testing. Since the preparation of Per a 2, Per a 5, and Per a 10 displayed a tendency of unspecific IgE binding, the threshold for test positivity was set to 0.35 kU$_A$/L for these allergens (**Figure 3B**). Other *Periplaneta* allergens were evaluated with a cutoff of 0.1 kU$_A$/L (**Figure 3A**). Although no major allergen could be identified, Per a 3 and Per a 9 appeared as the most important in this study. The frequencies of sensitization to the different allergens in the entire study population were as follows: Per a 1: 16% (16/98), Per a 3: 41% (40/98), Per a 4: 18% (18/98), Per a 7: 28% (27/98), Per a 8: 24% (23/98), and Per a 9: 50% (49/98) (**Figure 3A**). Per a 4 and Per a 8 were characterized as minor allergens in comparison with other *Periplaneta* allergens. The frequency of IgE recognition of Per a 2, Per a 5, and Per a 10 was 4, 19, and 21%, respectively (**Figure 3B**).

The sensitization pattern within each patient group was diverse and not comparable between the different patient collectives (**Figure 4**). Analysis of individual patient groups from different geographical areas revealed the highest prevalence of IgE reactivity to Per a 3 (59 and 67%) and Per a 9 (57 and 67%) in Indian and Korean patients. Per a 9 was the most prominent (45%) allergen also among the Iranian patients, whereas patients from Venezuela reacted predominantly to Per a 7 (31%) (**Figure 4**). Per a 4 and Per a 8 were identified as minor allergens in patients from almost all investigated geographies. However, the prevalence of sensitization to these components was the lowest among subjects from Venezuela. Patients from India and Korea showed low frequencies of IgE reactivity to Per a 2, Per a 5, and Per a 10 (**Figure 4**) (India: 3% to Per a 2, 28% each to Per a 5 and 10; Korea: 11% to Per a 2, 44% to Per a 5 and 48% to Per a 10). Notably, none of the Venezuelan or Iranian patients were sensitized to any of these three proteins (**Figure 4**).

Of the 95 *Periplaneta* ImmunoCAP-positive patients, 28 (29%) did not react with any of the purified allergens tested (**Supplementary Table 2**). This discrepancy was most prominent in serum samples from Iran 9/19 (47%), but less frequent in samples from India 6/28 (21%), Korea 7/27 (26%), and Venezuela 6/21 (29%). It is tempting to speculate that these patients are sensitized to other *Periplaneta* proteins or isoforms not reflected by the present CRD panel. In contrast, only 4% (5/117) of the patients showed IgE sensitization (with IgE levels close above the cutoff) to one or more of the purified allergen(s) despite a negative test result with *Blattella* and *Periplaneta* ImmunoCAP.

IgE Immune Reactivity of Periplaneta Extract

Sera of cockroach-sensitized patients ($n = 7$) from Venezuela either lacking any IgE reactivity (VR03, VR06, VR10, VR20) or showing very low IgE values of 0.12 kU$_A$/L (VR07, VR12)

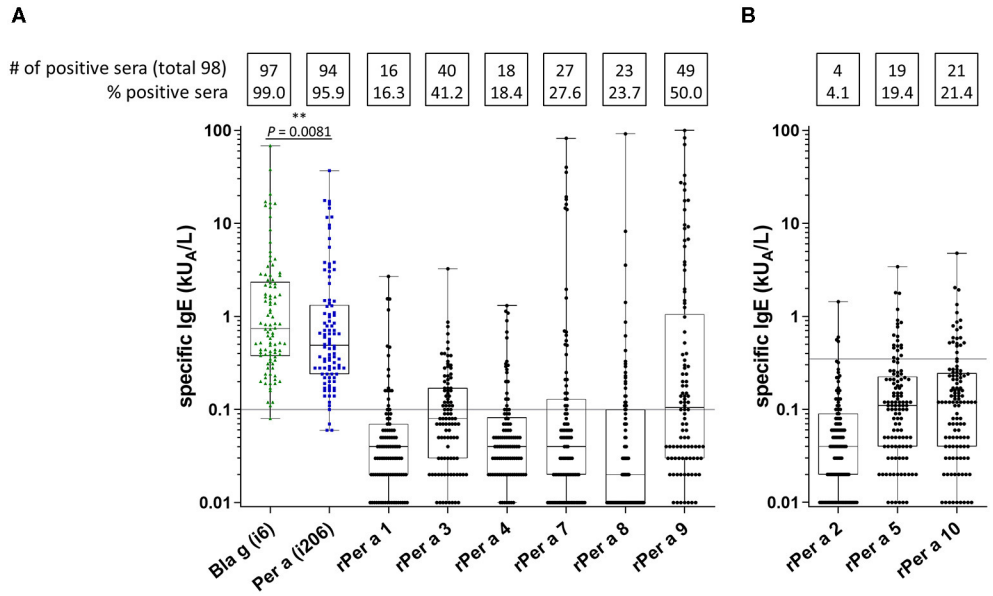

FIGURE 3 | CRD of cockroach allergy by experimental ImmunoCAP testing including cockroach-allergic patients preselected by positive *Periplaneta*- or *Blattella*-specific ImmunoCAP values ($n = 98$). Specific IgE values for *Periplaneta* and *Blattella* extracts and Per a 1, Per a 3, Per a 4, Per a 7, Per a 8, and Per a 9 (cutoff 0.1 kU_A/L) **(A)**, as well as for Per a 2, Per a 5, and Per a 10 (cutoff 0.35 kU_A/L) **(B)** are depicted. (paired *t*-test).

	Bla g (i6)	Per a (i206)	rPer a 1	rPer a 2*	rPer a 3	rPer a 4	rPer a 5*	rPer a 7	rPer a 8	rPer a 9	rPer a 10*
total (n = 98)	97 (99.0%)	94 (95.9%)	16 (16.3%)	4 (4.1%)	40 (41.2%)	18 (18.4%)	29 (20.4%)	27 (27.6%)	23 (23.7%)	49 (50.0%)	21 (21.4%)
Indian (n = 29)	29 (100%)	28 (96.6%)	3 (10.3%)	1 (3.4%)	17 (58.6%)	7 (24.1%)	8 (27.6%)	7 (24.1%)	8 (27.6%)	17 (58.6%)	8 (27.6%)
Korean (n = 27)	27 (100%)	27 (100%)	7 (25.9%)	3 (11.1%)	18 (66.7%)	7 (25.9%)	12 (44.4%)	9 (33.3%)	7 (25.9%)	18 (66.7%)	13 (48.1%)
Venezuela (n = 22)	22 (100%)	20 (90.9%)	4 (18.2%)	0 (0.0%)	2 (9.1%)	1 (4.5%)	0 (0.0%)	7 (31.8%)	3 (13.6%)	5 (22.7%)	0 (0.0%)
Iran (n = 20)	19 (95.0%)	19 (95.0%)	2 (10.0%)	0 (0.0%)	3 (15.0%)	3 (15.0%)	0 (0.0%)	4 (20.0%)	5 (25.0%)	9 (45.0%)	0 (0.0%)

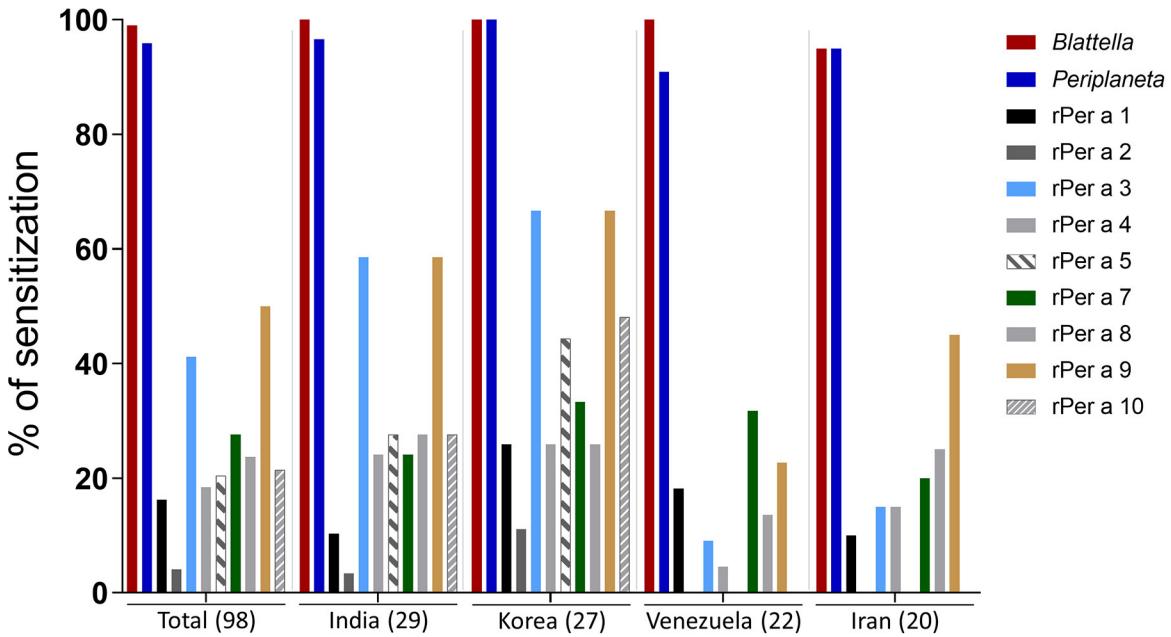

FIGURE 4 | Comparison of IgE antibody levels to *Periplaneta* (i206), *Blattella* (i6), and purified recombinant *Periplaneta* allergens in respective patient collectives from India ($n = 29$), Korea ($n = 27$), Venezuela ($n = 22$), and Iran ($n = 20$). Prevalence (%) of IgE sensitization to Per a 1, Per a 3, Per a 4, Per a 7, Per a 8, and Per a 9 (cutoff of 0.1 kU_A/L) and Per a 2*, Per a 5*, and Per a 10* (cutoff of 0.35 kU_A/L) are shown.

to purified allergens were further analyzed by immunoblotting using *Periplaneta* extract (**Figure 5**). Two sera (Sal and PL6) were applied as positive controls. IgE binding to putative novel and yet not identified *Periplaneta* proteins was demonstrated for six out of seven sera, showing IgE binding to distinct high molecular weight (HMW) proteins and proteins with an apparent molecular weight of 16 kDa (VR3, VR12), 22 kDa (VR12), and 40 kDa (VR6, VR9, and VR20) not verified so far.

However, the accumulation of both proteins, lipocalin and myosin light chain, in *Periplaneta* extract was confirmed by MS/MS analysis. Peptides derived from both allergens showed a sequence coverage of 15 and 29% (for Per a 4) and 34 and 59% (for Per a 8), in two extracts analyzed independently (**Supplementary Figure 3**).

DISCUSSION

Cockroaches are an important source of indoor aeroallergens. However, allergens from American cockroach (*P. americana*) are less investigated in comparison with German cockroach (*B. germanica*) allergens, and reports on the involvement of individual *Periplaneta* allergens in allergy related morbidity are inconsistent. Notably, clinically relevant sensitization to *Blattella* across Europe was reported in the GA^{2}LEN skin test study (29). In addition, sensitization to different cockroach species has been reported in South Italy (30), demonstrating positive RAST results to *Periplaneta* in four of 15 cockroaches (mixed species) SPT-positive patients (31). However, in Italian children, sensitization was substantially higher to *Periplaneta* than to *Blattella* (32). So far, the molecular sensitization profile to *Periplaneta* allergens was not addressed in these studies, except one study describing reactivity to Per a 7 (tropomyosin) in 41% of *Periplaneta*-sensitized indoor allergic patients from Marseille (19). All these studies implicate that cockroach allergen exposure and sensitization are a global health problem that will likely increase along with global warming. This prompted us to investigate the involvement of *Periplaneta* allergens in the sensitization profile to cockroach.

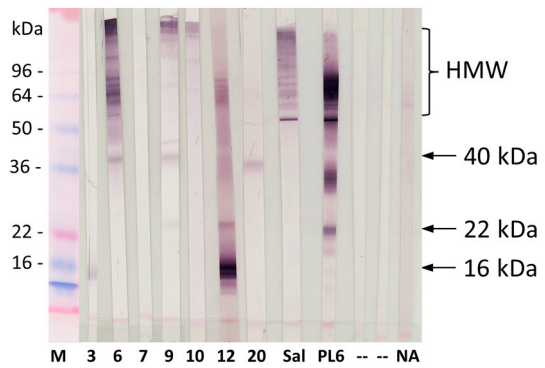

FIGURE 5 | IgE-reactivity pattern of cockroach-reactive patients from Venezuela ($n = 7$, VR3, VR6, VR7, VR9, VR10, VR12, VR20) showing negative ImmunoCAP results for recombinant *Periplaneta* allergens, Germany (Sal), and pooled sera from cockroach-sensitized patients (PL6) with *Periplaneta* extract. M, marker; P, Ponceau S, staining; NA, non-atopic control; "-", secondary antibody control. Arrows indicate the putative yet not identified allergens.

In the present study, using different patient collectives, a substantial panel of recombinant *Periplaneta* allergens, including yet less well-characterized allergens Per a 4 and Per a 8, was utilized for CRD employing a uniform analytical methodology. The identity of all purified *Periplaneta* allergens used in the study was confirmed by MS. We attempted to produce Per a 6 but were unable to confirm its identity. Three known *Periplaneta* allergens Per a 11 to Per a 13 were not included in the study since they were not yet been identified at the time the study was initiated.

A total of 117 patients from four countries with subtropical or tropical climates, with perennial respiratory indoor allergy, and evidence of IgE sensitization to cockroach (evaluated by *in vitro* and *in vivo* methods), were included in the study. Of note, the limitation of the study is the lack of consistent inclusion criteria in terms of clinical cross-reactivity (co-sensitization with cross-reactive mite and insect allergens), and the application of different IgE detection methods in different cohorts, which can lead to a bias of the preselected patients. Cockroach-specific IgE was re-evaluated and confirmed in 81% and 83% of all sera by commercial ImmunoCAP testing, using *Periplaneta* or *Blattella* extracts, respectively. Whereas, sera from Korean patients were preselected by positive ImmunoCAP results, IgE sensitization of patients from Venezuela, India, and Iran was assessed by either skin testing, ELISA, or immunoblotting, showing a correlation with *Periplaneta*-positive ImmunoCAP testing between 63 and 80%. Discrepancy between results from ImmunoCAP and other test systems might be due to various applications of non-standardized and self-prepared extracts, as well as the different diagnostic sensitivity of each test system.

ImmunoCAP testing showed an almost similar diagnostic sensitivity for *Blattella* and *Periplaneta* extract and levels of IgE to the two cockroach species were strongly correlated ($r = 0.95$). Only one patient with low *Periplaneta*-specific IgE but negative *Blattella*-specific IgE values was detected. *Vice versa,* four patients with reactivity to *Blattella*, but without sensitization to *Periplaneta*, were identified. Finally, the diagnostic sensitivity using recombinant allergens was increased for five patients who did not react with any of the two extracts. The overall concordant results are likely explained by the presence of conserved and cross-reactive allergens in *Periplaneta* and *Blattella*, consistent with the official recognition of allergens from at least seven groups identified in both species. However, only partial IgE cross-reactivity between members of homologous groups has been reported (2). Slight variability of IgE-binding capacity between the species might be due to distinct expression levels of certain allergens in cockroaches, a variable presence of allergens in the applied extracts, or the abundance of species-specific allergens. In line with this, group 4 and 8 allergens were already denominated according to the IUIS allergen nomenclature database for *Blattella* but not for *Periplaneta*, whereas group 10 and 13 allergens have so far not been identified in *Blattella*. In the present study, we produced recombinant *Periplaneta* proteins homologous to Bla g 4 and Bla g 8 and included them in the panel of allergens used for CRD.

Component-resolved diagnostics was performed with patients ($n = 98$) reporting perennial respiratory indoor allergies and IgE sensitization to *Blattella* or *Periplaneta,* confirmed by ImmunoCAP testing as a uniform methodology across the entire

population. Results showed only 68% (67/98) of ImmunoCAP-positive patients to react with any of the nine recombinant *Periplaneta* allergens tested. However, a study addressing CRD of *Blattella* allergy suggested a panel of only four allergens (Bla g 1, Bla g 2, Bla g 4, and Bla g 5) to be sufficient to identify 95% of cockroach-allergic patients in the United States (33). In another study, all patients with airway allergy and positive *Blattella* ImmunoCAP values were reported to react with at least one of Per a 1 to 7 and Per a 9, tested by ELISA (12). In contrast, our study is in agreement with other reports demonstrating that a set of several recombinant *Blattella* allergens fall short of reflecting the IgE reactivity of corresponding natural *Blattella* allergen extract (34, 35). By applying a panel of allergens, only 49% and 62% of cockroach-allergic patients were tested positive either by skin testing (35) or by *in vitro* assays (34), respectively. Both studies are in accordance with our present results showing a higher diagnostic sensitivity of the natural extract in comparison with the applied recombinant allergens. The higher diagnostic value of cockroach extract in comparison with the panel of purified allergens can be explained by missing allergens which need to be included in CRD, structural modification of recombinant allergens affecting the IgE reactivity (e.g., mediated by posttranslational modifications, or partial aggregation of recombinants). Furthermore, the presence of IgE-reactive natural variants/isoforms that are not reflected by the selected recombinant allergens could play a role in the lower diagnostic sensitivity of the CRD.

The present study demonstrated a heterogeneous pattern of IgE sensitization to *Periplaneta* allergens in different patient collectives. The IgE-reactivity profiles could be in part due to the exposure and IgE responses to abundant cross-reactive homologous allergens. For all *Periplaneta* allergens except Per a 1, homologous groups and potential cross-reactive allergens have been described among invertebrates other than cockroaches (1). In agreement with a previous notion by Pomes et al. (1), we did not observe any dominant and thus major *Periplaneta* allergen considering the whole study collective. Importantly, the analysis of the individual geographical different cohort revealed Per a 3 and Per a 9 as major allergens (with IgE prevalence $\geq$50% in the respective patient group) in India and Korea. This, together with the occurrence of extract-positive subjects without IgE to any of the components included in this study, indicates the need to identify and evaluate additional *Periplaneta* allergens in each cohort.

In the present study, lipocalin and myosin light chain were classified for the first time as minor *Periplaneta* allergens in all investigated cohorts with an overall frequency of IgE sensitization of 18 and 24%, respectively. Both proteins were accepted by the WHO/IUIS Allergen Nomenclature Sub-Committee as Per a 4 and Per a 8. Sensitization to recombinant Per a 4 was most prevalent in patients from Korea (26%), and sensitization to recombinant Per a 8 was most prevalent in Indian patients (28%). Whether the IgE-binding capacity of recombinant allergens is reflected by the natural counterparts remains open. However, our study provided evidence that the prominence of allergens is different in the respective patient collective. The results are in agreement with a limited number of studies describing molecular sensitization profiles to *Periplaneta* allergens and showing a heterogeneous clinical significance of single allergens. In line with this, the prevalence of IgE sensitization of 5–100% for Per a 1, 63% for Per a 2, 26–95% for Per a 3, 30–70% for Per a 5 (16), 13–54% for Per a 7, 80–100% for Per a 9, 82% for Per a 10, 83% for Per a 11, and 64% for Per a 12 has been reported (7). A retrospective study of 118 cockroach-sensitized subjects from inner-city environments in the United States revealed that only 13% were sensitized to Per a 7 (34). In contrast, Per a 7 was found to be the dominant allergen among 55 cockroach-allergic patients in Brazil, with a prevalence of IgE reactivity of 42% (35).

Our data suggested the involvement of yet unidentified *Periplaneta* allergens (including isoforms and variants) and/or a potential important role of Per a 6, Per a 11 to Per a 13, which were not tested in the current study. Moreover, 2D-immunoblotting and subsequent MS analysis using pooled sera, not reactive to any of the tested recombinant Per a allergens, revealed IgE binding to 12- and 16-kDa proteins, in addition to a 40-kDa band which might correspond to Per a 11 to Per a 13 (data not shown). In addition, the role of protein glycosylation in the immunogenicity of cockroach allergy (2) needs to be considered. Insects express immunogenic core α-1,3 carbohydrate structures with additional −1,6 fucosylation (36), and glycan modification of *Blattella* allergens has been described (37, 38). Although reports on cross-reactive carbohydrate determinants (CCD) of cockroach allergens are limited, and routine diagnosis lacks insect-specific glycan moieties, it is tempting to speculate that a substantial number of cockroach-sensitized patients are reactive to glycan structures which in part may explain our observed gap in diagnostic sensitivity between natural cockroach extracts and a panel of recombinant, non-glycosylated allergens.

In summary, we show that levels of IgE antibodies to *Periplaneta* and *Blattella* are strongly correlating and that natural *Periplaneta* extract displays higher diagnostic sensitivity than a panel of nine recombinant *Periplaneta* allergens. For reasons that are elusive, patient collectives from different countries showed heterogeneous sensitization profiles. Notably, major allergens could be identified only for individual cohorts, while Per a 4 and Per a 8 were identified as minor allergens. Further improvement of *Periplaneta* CRD requires exploration of the role of isoforms, glycan determinants, and additional yet unknown allergens, which are likely classified already in other insects.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical approval was obtained from IHEC (no. CLP 0019, CSIR-IGIB) and Isfahan university of Medical Sciences and Health

services (no. 295264). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JS, RR, RS, SSa, FT, J-WP, and NA recruited and characterized the patients. Recombinant Per a 1, Per a 2, and Pera 3 were prepared and characterized by SE and IP. Per a 4, Per a 7, Per a 8, and Per a 9 by AW and AJ. Per a 5, Per a 6, and Per a 10 by SSh. Protein and MS analysis were performed by BS, BK, SW, GG and PB. Experimental ImmunoCAPs were established and performed by JL and FFü. NA, FFe, and SV were responsible for the study. GG, AW, and SSc coordinated the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

This study was funded by ERAnet New INDIGO Project GENALL DBT2-038. The authors wish to thank Ulrica Olsson for preparing experimental ImmunoCAP tests and Elke Haberkorn for excellent technical support and conducting the ImmunoCAP measurements.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2021.691627/full#supplementary-material

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Conflict of Interest: JL was employed by company Thermo Fisher Scientific.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Component-resolved diagnosis of American cockroach (*Periplaneta americana)* allergy in patients from different geographical areas

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Keywords

Cockroach allergy, American cockroach, *Periplaneta americana*, *Blattella germanica*, component-resolved diagnosis

Supplementary data

Material and Methods

Preparation of *Periplaneta americana* extract

Periplaneta americana allergen extract was prepared as described previously (1) (2). Briefly, freeze-dried (adult, whole body) cockroaches were powdered, defatted with diethyl ether and antigens were extracted (1:50 (w/v)) in 0.1 M phosphate buffer saline (PBS), pH 7.2, containing 5 mM EDTA (ethylenediaminetetraacetic acid; Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), by continuous stirring for 8 h at 4°C. Supernatant was collected after centrifugation (10-12.000 rpm), dialysed against A.d., and lyophilized. Whole protein concentration was estimated by BCA Assay (bicinchoninic acid, Sigma). Finally the extract was subjected to ELISA or was used for immunoblotting experiments to verify IgE-binding to *Periplaneta* allergens.

To verify the accumulation of additional allergens by immunoblotting (Fig. 5) commercial ground whole body powder of *Periplaneta americana* cockroaches (Allergon, Thermo Fisher Scientific, Ängelholm, Sweden) was mixed in PBS (0.8 g/10 ml) using an Ultra-Turrax ®(IKA, Staufen, Germany) and incubated overnight (4°C) on an end over end rotator (Reax2, Heidolph-Instruments, Schwabach, Germany). The protein extract was prepared by centrifugation (12.000xg, 30', 4°C) and filtration (5 µm, 0.8 µm and 0.45 µm). Whole protein content was determined by Roti-Nanoquant 5× Bradford reagent (Carl Roth, Karlsruhe, Germany).

Generation of recombinant *Periplaneta* allergens

Per a 1

The sequence of Per a 1 was obtained from UniProt (O18530). The predicted/putative signal peptide was not considered, and a glycine was inserted after the start-methionine for stabilization of the protein. In addition, the sequence was adapted to destroy an internal XhoI resitriction site (T576G). The gene was synthesized and cloned into pHIS-Parallel2 vector using NdeI and XhoI restriction sites by ATG:biosynthetics GmbH (Merzhausen, Germany) (3). Per a 1 consists of two repeats and is highly prone to degradation. Previous work on the homologue Bla g 1 focused on the second repeat only (4), which is why we sub-cloned Per a 1

repeat 2 (residue 197-378) using 5'-GAG ACA TAT GGG TCG CAA TCT GCA A-3' as forward and 5'-TCT CCT CGA GTC AGA ACA AGC TGC G-3' as reverse primer. Protein expression was performed in *E. coli* BL21Star at 37°C for 3 hours after induction with 0.4 mM IPTG. Harvested bacteria were dissolved in 10 mM NaOAc pH 8.6, 10 mM EDTA and broken by repeated freeze and thaw cycles. The first purification step involved an anion exchange chromatography using a 5 ml HiTrap DEAE column and the ÄKTA Prime purification system (GE Healthcare). Proteins were eluted with 1 M NaCl buffer and fractions containing enriched Per a 1 were pooled. Buffer exchange to 2 mM Tris pH 8.6 and concentration of the protein sample was achieved using an Amicon Ultra-15 Centrifugal Filter with 3 kDa cut-off (Millipore). Finally, size exclusion chromatography using a Superdex 75 10/300 GL column connected to an ÄKTA FPLC system (GE Healthcare) was performed. Eluting proteins were supplemented with 2 mM PMSF and fractions containing non-tagged Per a 1 repeat 2 were pooled.

Per a 2

The mature protein sequence of Per a 2 (Q1M0Y6) was ordered at ATG:biosynthetics (Merzhausen, Germany) and sub-cloned into the pHisParallel2 vector (40) with NdeI and XhoI restriction sites using 5'-GAG ACA TAT GGG TGA TCC AGT CGT CGT-3' and 5'-TCT CCT CGA GCA GTT CTT CTA CGG A-3'. A glycine was introduced at the N-terminus for enhanced protein stability and the protein was constructed for expression with a C-terminal His_{tag}. Protein expression was conducted in *E. coli* SHuffle (Novagen, Gibbstown, NJ, USA). The bacterial culture was induced with 0.4 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and grown at 10°C for 48 hours. Cells were harvested by centrifugation at 4,000 g for 20 min and cells were lysed by three cycles of freezing and thawing followed by sonication in 10 mM sodium phosphate pH 7.0, 1 M NaCl and 6 M urea (lysis buffer). Supernatants after centrifugation at 14.000 g for 30 min were filtered and loaded onto a 1 mL His-Trap HP column (GE Healthcare, Little Chalfont, UK) using the ÄKTA Protein Purification Systems (GE Healthcare). A washing step of 30 ml lysis buffer was performed followed by an on-column refolding gradient of 400 ml to 10 mM sodium phosphate pH 7.0, 1 M NaCl. Proteins were gradient eluted using 10 mM sodium phosphate pH 7.0, 1 M NaCl, 500 mM imidazole.

Per a 3

The gene corresponding to Per a 3.0101 (Q25641) was ordered at ATG:biosynthetics (Merzhausen, Germany) and the Per a 3 C-domain harboring the IgE binding epitopes sub-

cloned into the pHisParallel2 vector (40) with NdeI and XhoI restriction sites using 5'-GAG ACA TAT GGG TCC CCG TTA C' and 5'-TCT CCT CGA GCT TGT GGA AGA T-3'. The construct was transformed into *E. coli* Rosetta-gamiB pLysS and expression was induced with 0.4 mM IPTG. Recombinant expression of the C-terminal His_{tag} protein was conducted at 16°C overnight and cell harvest and disruption performed by freeze and thaw cycles (see Per a 1). The bacterial pellet was first dissolved in 10 mM Na_2HOP_4 pH 7.4, 250 mM NaCl, 10 mM imidazole and 6 M urea, the resulting pellet after centrifugation was dissolved again in 10 mM Na_2HOP_4 pH 7.4, 250 mM NaCl, 10 mM imidazole and 0.5% SDS. The protein extract was loaded on a 5 ml HisTrap FF crude column and elute using 10 mM Na_2HOP_4 pH 7.4, 250 mM NaCl, 500 mM imidazole, 0.1% sarkosyl. For refolding purpose and to keep the protein in solution, Per a 3 C-domain was buffer exchanged to 10 mM Na_2HOP_4 pH 7.4, 0.1% sarkosyl. For stability in long-time storage, 20 mM EDTA and 10 mM PMSF were added to the final pool.

Per a 4:

Per a 4 (AY792948) without signal peptide was generated in *E. coli*. A codon optimized synthetic gene (GeneArt, Thermo Fisher Scientific) was subcloned into expression vector pET11a. The Per a 4/pET11a construct was transformed to Origami2(DE3) *E. coli* cells, which were used for protein expression (3 L in Luria Broth (LB) low salt medium) using isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, at an optical density (OD_{600}) of 0.7 for induction of protein expression over night at 22°C. Cells were harvested by centrifugation (20', 5000xg) and lysed using 20 mM Bis-Tris (pH 5.8) by three times freeze/thawing cycles under liquid nitrogen. Lysate was prepared by centrifugation (30 min, 13,000xg, 30 min, 4°C) and filtration (5 µm, 0.8 µm, 0.45 µm). For purification of recombinant Per a 4 the lysate was applied to two step anion exchange chromatography (Hi Prep DEAE FF 16/10 followed by HiPrepQ HP 16/10, GE Healthcare, Freiburg, Germany). Protein concentration of Per a 4 was measured using the BCA protein assay (Thermo Fisher Scientific).

Per a 5

For cDNA cloning of Per a 5 gene freeze dried cockroach powder was utilized for total RNA isolation using EZ-RNA isolation kit (Biological Industries Sartorius) and converted to cDNA by RevertAid(H-) cDNA synthesis kit (Thermo Fisher Scientific). Per a 5 gene (AY792949.1) was PCR amplified using gene specific primers. The PCR product and vector backbone were cleaved by Eco R I and Xho I, gel eluted by QIAquick gel extraction kit (Qiagen, Germany) and ligated

using T4 DNA Ligase (Thermo Fisher Scientific) at 16 ºC overnight. Ligation mixture was transformed in *E.coli* DH5α cells and positive colonies were grown overnight at 37ºC. Restriction digestion of positive colony showed the presence of 651 bp insert. The clone was also confirmed by gene sequencing. Forward primer: 5'-GTA TTG GAA TTC GAT GAC CAT CGA CTT CTA C-3'. Reverse primer: 5'-ACT GAA CTC GAG CTT CTT GGC GAG GTT ATC-3'. Recombinant Per a 5 (rPer a 5) was expressed in *E.coli* BL21-λ DE3 cells. Briefly, the clone containing Per a 5 was grown until OD_{600} reached 0.5 and induced with 1mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h followed by harvesting. The cell pellets obtained were dissolved in binding buffer (50 mM Tris, 300 mM NaCl), sonicated and centrifuged. Meanwhile, Ni-NTA agarose (Qiagen, Germany) resin was equilibrated with binding buffer and incubated with supernatant for 2 h at 4ºC. The column was washed with wash buffer (binding buffer with 25 mM imidazole) and eluted with elution buffer (binding buffer containing 250 mM imidazole). Protein elutes were concentrated and dialyzed with Amicon concentrator (Millipore) and re-purified with Ni-NTA resin. The re-purified elutes were concentrated, estimated (by BCA assay) and resolved as a single band at approximately 27 kDa on 12% SDS-PAGE.

Per a 7, Per a 8 and Per a 9: *E. coli* optimized synthetic genes (GeneArt, Thermo Fisher Scientific). for Per a 7.0101 (Y14854), Per a 8 (JQ279816) and Per a 9 (AY563004) were subcloned into expression vector pET23, and transformed into BL21(DE3) *E. coli* cells. Protein expression of 2 L (Per a 7, Per a 9) or 8 L (Per a 8) *E. coli* culture was induced by addition of IPTG to a final concentration of 1 mM, at an optical density (OD_{600}) of 0.7. After 16 hours at 22°C, the cultures of Per a 7 and Per a 8 were harvested and the cells lysed in 20 mM Bis-Tris (pH 6) containing 4 M urea for 30 min at room temperature on an end over end rotator (Reax2, Heidolph-Instruments). The recombinant proteins were purified from the supernatant of the lysates by two step anion exchange chromatography (Hi Prep DEAE FF 16/10 followed by HiPrepQ HP 16/10, GE Healthcare) using on column refolding on DEAE column with a 4-0 M urea gradient followed by a 0-1 M NaCl gradient for elution, both in 20 mM Bis-Tris (pH6). Elution fractions containing the recombinant proteins were pooled and applied to size exclusion chromatography (HiLoad 26/600 Superdex 75 pg, GE Healthcare) using 20 mM MOPS, 0.5 mM NaCl (pH 7) as running buffer. For purification of rPer a 9 the cells were lysed in 20 mM Tris (pH 8) by three times freeze/thawing cycles under liquid nitrogen. Lysate was prepared by centrifugation (30 min, 13,000 g, 30 min, 4°C) and filtration (5 µm, 0.8 µm,

0.45 µm). Subsequently the lysate was applied to anion exchange chromatography (MonoQ HR5/5, GE Healthcare, Freiburg, Germany) followed by size exclusion chromatography (HiLoad 26/600 Superdex 75 pg, GE Healthcare) using 20 mM MOPS, 0.5 mM NaCl (pH 7.6) as running buffer as final polishing step. Protein concentration of the recombinant proteins was measured by the BCA protein assay (Thermo Fisher Scientific).

Per a 10

For cDNA cloning of Per a 10 total RNA was isolated from freeze-dried *Periplaneta americana* using EZ-RNA kit (Biological Industries, Kibbutz Beit-Haemek, Israel) and cDNA synthesized as per manufacturer's protocol (Qiagen-Sensiscript RT Kit, Hilden, Germany). cDNA was amplified by gene specific primers SP 1 5'-GTA TTG GAATTC ATG CTT CGC TAC CTG GTA-3' and SP 2 5'-ACT GAA GCT CGA GGT TAG TTG ACT CAG TCT GTT CTG-3' with Eco RI and Xho 1 sites, respectively, at flanking ends. PCR conditions include initial denaturation at 95°C for 5 min and then 30 cycles of 94°C 1 min, 55°C 1 min, 72°C 1 min, with final extension at 72°C for 7 min. PCR product and pET22b+ vector were digested with Eco RI and Xho 1 restriction enzymes, ligated, then transformed into *E. coli* DH5α cells and positive clones were confirmed by restriction digestion. The clone was sequenced at The Centre for Genomic Application, Delhi and designated as pSP10. For recombinant expression of rPer a 10 *E. coli* BL21 cells having pSP10 were grown until optical density 0.6, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Fluka, Sigma, St. Louis, MO, USA) for 4 h and cells harvested at 7600 RCF for 10 min at 4°C. The cell pellet was resuspended in binding buffer (50 mM Tris, 300 mM NaCl and 10 mM imidazole; pH 8.0), sonicated and centrifuged for 30 min at 4°C. The supernatant was incubated with 1 mL Ni-NTA resin (Qiagen) for 1 h at 4°C and washed with 20 mM imidazole in binding buffer. His_{tag} protein was eluted using 250 mM imidazole, desalted and concentrated using Amicon Ultra-15 (Millipore, Billerica, MA, USA). Recombinant Per a 10 (rPer a 10) amount was estimated (by BCA assay) and polyacrylamide gel electrophoresis performed under non-reducing conditions to determine the homogeneity (5).

Physicochemical characterization of *Periplaneta* allergens

For consistent analyses, all purified recombinant proteins were re-buffered to 10 mM of potassium phosphate pH 7.0 using centrifugal filter devices (Ultra-Amicon) (Millipore corporation, Billerica, US), by several rounds of centrifugation at 12.000 rpm.

Amino acid analysis

Amino acid analysis (AAA) was performed with all proteins in duplicates following the Pico-Tag method (Waters, Milford, MA, USA). Phenylthiocarbamyl amino acid derivatives were analyzed by reversed phase high-performance liquid chromatography (UltiMate 3000, Thermo Fischer Scientific, Waltham, MA, USA), using a 3.0x150 mm XSELECT™ HSS T3 3.5 µm column (Waters). Hydrolyzed amino acids were quantified at 254 nm by peak area comparison to the amino acid standard H (Pierce, Rockford, IL, USA).

SDS-PAGE analysis

Based on the protein concentration obtained by AAA, 2 µg of all recombinant allergens were loaded on 15% polyacrylamide gels under reducing conditions. Proteins were visualized with Coomassie Brilliant Blue R-250 staining (Bio-Rad, Hercules, CA, USA). Images were taken with ChemiDoc MP Imaging System (Bio RAD) and acquired with the software Image Lab. 4.0.1.

Mass spectrometry analysis

For intact mass measurements of purified allergens, the samples were desalted with C_{18} ZipTips (Merck, Millipore, Billerica, MA, USA) and directly infused into the Q-Exactive mass spectrometer (Thermo Fisher Scientific) at a flow rate of 1 µL/min, using the nano-electrospray head. Raw data obtained from intact proteins were processed with Protein Deconvolution 2.0 (Thermo Fisher Scientific). Additionally, intact mass measurements were performed with reduced and alkylated proteins. The samples were reduced and alkylated with reducing and blocking buffer from the ProteoExtract All-in-One Trypsin Digestion Kit (Merck Millipore, Billerica, USA) and measured as described above. For peptide mapping analysis 1 µg of each protein was reduced, alkylated and digested with the mentioned Trypsin Digestion Kit. Resulting peptides were separated by reverse-phase nano-HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific). Peptides were loaded onto the trap column (PepSwift Monolithic Trap Column, Dionex) and desalted with 0.1% (v/v) heptafluorobutyric acid at a flow rate of 10 µL/min. After 5 min, trap and separation column (PepSwift Monolithic Nano Column, 100 µm x 25 cm, Dionex) were coupled with a switching valve and the peptides were eluted with an acetonitrile gradient (Solvent A: 0.1% (v/v) Formic acid (FA), 0.01% (v/v) Trifluoroacetic acid (TFA), 5% (v/v) Acetonitrile (ACN); solvent B: 0.1% (v/v) FA, 0.01% (v/v) TFA, 90% (v/v) ACN; 5–45% B in 60 min) at flow rate of 1 µL/min at 55°C. The HPLC was directly coupled via nano-electrospray to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific).

Capillary voltage was 2 kV. For peptide identification, a top 12 method was used. The normalized fragmentation energy was 28%. Survey and fragment spectra were analyzed with Proteome Discoverer version 1.4 (Thermo Fisher Scientific) or Peaks Studio 7.5 (Bioinformatics Solutions, Waterloo, Canada), respectively.

Circular dichroism and dynamic light scattering

Circular dichroism spectra to study the secondary structure of all purified allergens were recorded in 10 mM of potassium phosphate pH 7.0 with a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan). Far UV spectra (190 - 260 nm) were baseline subtracted and results are presented as mean residue molar ellipticity. Three-dimensional structure model of all allergens were generated using SWISS-MODEL (https://swissmodel.expasy.org/). The global model quality estimate (GMQE) was considered for the quality of the models. Three-dimensional structure representations were generated with Chimera 1.8 (www.cgl.ucsf.edu/chimera).

The aggregation behavior of the proteins was analyzed by dynamic light scattering (DLS). DLS analyses were performed with protein concentrations of 1 mg/mL and the data acquisition (10 measurements of 5 seconds duration) was done using DLS802 system (Viscotek Corp). Three independent measurements were performed for each protein and the results are presented as mean of three experiments.

Figure legends

Table S1: Identity and physicochemical characterization of recombinant *Periplaneta* allergens.

Table S2: *Periplaneta*-, *Blattella* extract and recombinant *Periplaneta* allergen specific ImmunoCAP values of 117 patients enrolled in the study (I: India; II: Korea; III: Venezuela; IV: Iran). Cut-off 0.1 kU_A/L, except for * (cut-off 0.35 kU_A/L).

Fig. S1: Secondary structure analysis of *Periplaneta* allergens. Circular dichroism spectra (190 – 260 nm) of purified allergens measured at 20°C. Three-dimensional structure models generated with SWISS-MODEL and presented with Chimera 1.8.

Fig. S2: Dynamic light scattering analyses of recombinant *Periplaneta* allergens. Rh, hydrodynamic radius in nm.

Fig. S3: Identification of Per a 4 and Per a 8 by MS/MS analysis of *Periplaneta* extracts (protein extraction was performed twice). Number and identity of peptides, as well as % sequence coverage and potential amino acid modification are indicated.

Fig. S4: Comparison of IgE antibody levels to *Periplaneta* (i206) and *Blattella* (i6) in the respective patient collectives from India (n=35), Korea (n=27), Venezuela (n=25) and Iran (n=30).

Fig. S5: CRD of cockroach allergy by experimental ImmunoCAP testing in country subgroup of patients (India: n=29, Korea: n=27, Venezuela: n=22 and Iran: n=20) preselected by positive *Periplaneta* or *Blattella*-specific ImmunoCAP values. *Periplaneta* (i206) and *Blattella* (i6) extract, and *Periplaneta* allergen specific IgE values for each patient are depicted. Cut-off 0.1 kU_A/L, except for * (cut-off 0.35 kU_A/L).

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Tab. S1

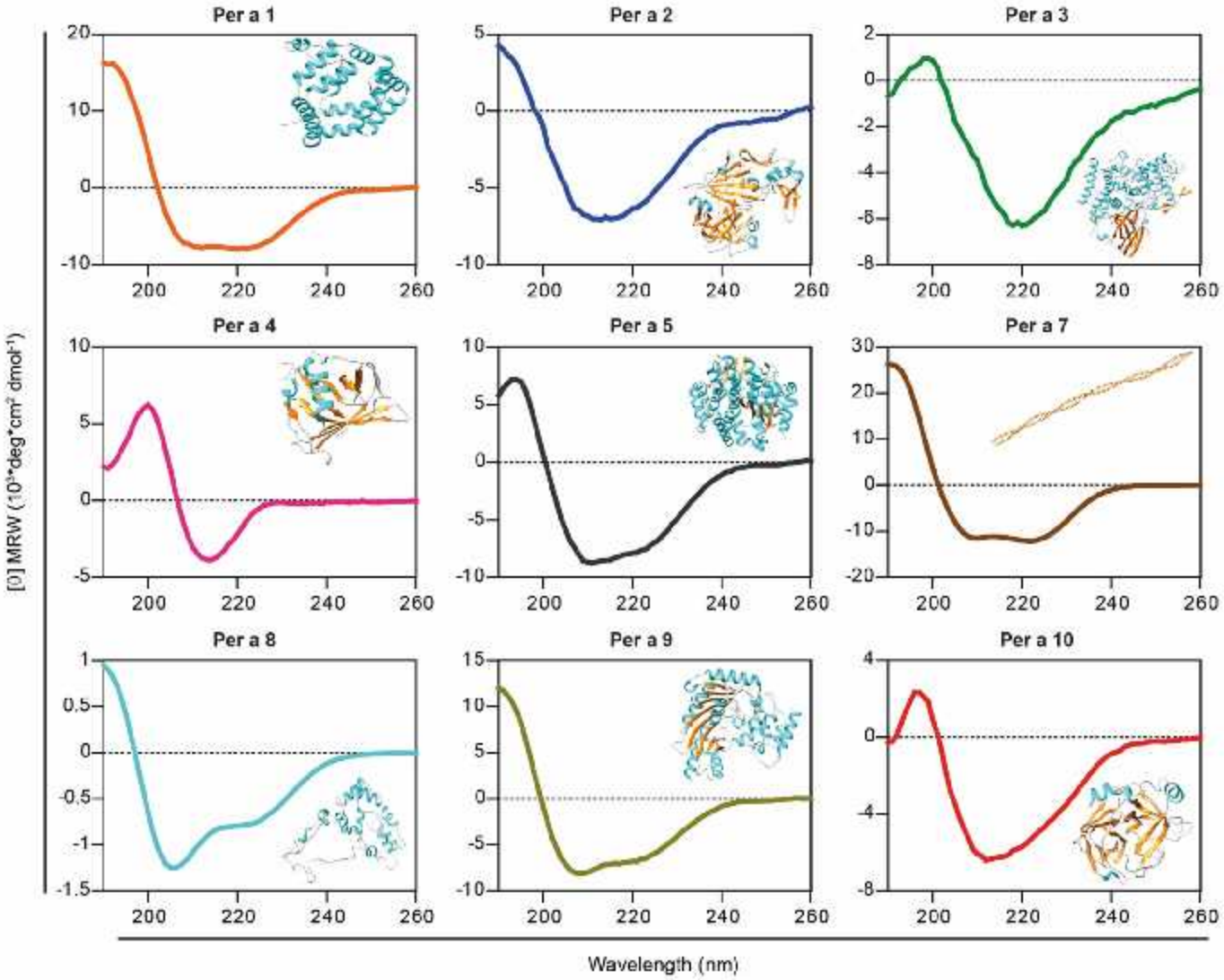
protein	UniProt ID	Modification	MW (kDa)	storage buffer	storage condition	c (µg/ml) from AAA	SDS-PAGE	MS – intact and reduced/alkylated (R/A) intact	MS - tryptic digestion (sequence coverage)	CD	DLS
Per a 1	O18530	repeat 2, none	20.9	2 mM Tris 10 mM EDTA 2 mM PMSF	-20°C in solution	270	detected	identity confirmed; R/A not performed (no cysteines)	identity confirmed by in-solution digest (98%)	folded	partially aggregated
Per a 2	Q1M0W6	C-terminal His-tag	26.9	10 mM NaP pH 7.0 1 M NaCl 0.5 M imidazole	+4°C in solution	167	detected	no signal when measured untreated intact; identity confirmed by intact R/A	identity confirmed by in-solution digest (60%)	folded	partially aggregated
Per a 3	Q25641	C-domain/C-terminal His-tag	29.5	10 mM Nap pH 7.0 10 mM EDTA 0.2% sarkosyl	-20°C lyophilized	498	detected	not possible due to detergent in the solution	identity confirmed by in-gel digest (78%)	partially folded	partially aggregated
Per a 4	Q1M0Y5	none	28.0	20 mM MOPS 500 mM NaCl	+4°C in solution	1155	detected	identity confirmed by intact and intact R/A	identity confirmed by in-solution digest (68%)	folded	mostly monomeric
Per a 5	Q1M0Y4	C-terminal His-tag	25.5	50 mM Tris 300 mM NaCl 250 mM imidazole	-20°C lyophilized	707	detected	not detectable	identity confirmed by in-solution digest (66%)	folded	partially aggregated
Per a 6	Q1M0Y3	N terminal His-tag	27.9	100 mM NaP 10 mM Tris 8 M Urea 250 mM imidazole	-20°C lyophilized	3.9	detected	not detectable	*identity not confirmed* (0%)	-	-
Per a 7	Q9UB83	none	32.8	20 mM MOPS 500 mM NaCl	+4°C in solution	486	detected	identity confirmed by intact and intact R/A	identity confirmed by in-solution digest (75%)	folded	homogenous size, fiber formation
Per a 8	H5WP59	none	22.5	20 mM MOPS 500 mM NaCl	+4°C in solution	1116	detected	identity confirmed, truncations at the N terminus, ~10% full length protein; R/A not performed (no cysteines)	identity confirmed by in-solution digest (90%)	folded	mostly monomeric
Per a 9	A1KY39	none	39.8	20 mM MOPS 500 mM NaCl	-20°C in solution	518	detected	identity confirmed by intact and intact R/A	identity confirmed by in-solution digest (74%)	folded	mostly monomeric
Per a 10	Q1M0X9	C-terminal His-tag	23.4	50 mM Tris 300 mM NaCl 250 mM imidazole	-20°C lyophilized	381	detected	not detectable	identity confirmed by in-solution digest (34%)	folded	partially aggregated

Tab. S2-I

Table S2-I: *Periplaneta-*, *Blattella* extract and recombinant *Periplaneta* allergen specific ImmunoCAP values of sera from India.

		ImmunoCAP™ [kU$_A$/L]											
#	internal #	Bla g (i8)	Per a (i206)	total IgE	rPer a 1	rPer a 2*	rPer a 3	rPer a 4	rPer a 5*	rPer a 7	rPer a 8	rPer a 9	rPer a 10*
1	1406_IGIB_1	0,39	0,33	96,0	0,02	0,04	0,08	0,04	0,10	0,01	0,01	1,97	0,12
2	1406_IGIB_2	0,35	0,18	49,1	0,04	0,05	0,11	0,05	0,10	0,03	0,02	0,05	0,12
3	1406_IGIB_3	0,16	0,20	131	0,05	0,04	0,07	0,03	0,12	0,02	0,01	0,04	0,16
4	1406_IGIB_4	0,05	0,06	8,31	0,02	0,02	0,06	0,03	0,05	0,02	0,01	0,04	0,12
5	1406_IGIB_5	2,17	0,27	25,3	1,18	0,05	0,10	0,04	0,11	0,06	0,01	0,04	0,14
6	1406_IGIB_6	20,70	17,30	734	0,07	0,08	0,17	1,14	0,32	40,10	1,42	9,23	0,33
7	1406_IGIB_7	0,31	0,22	120	0,03	0,04	0,09	0,04	0,17	0,04	0,02	0,15	0,17
8	1406_IGIB_8	0,05	0,03	49	0,02	0,02	0,05	0,02	0,06	0,01	0,01	0,01	0,07
9	1406_IGIB_9	0,51	0,45	396	0,03	0,06	0,12	0,04	0,23	0,06	8,21	27,40	0,25
10	1406_IGIB_10	0,84	0,65	375	0,02	0,08	0,11	0,05	0,25	0,03	0,02	0,28	0,27
11	1406_IGIB_11	0,07	0,06	23,2	0,04	0,06	0,13	0,05	0,12	0,03	0,02	0,06	0,14
12	1406_IGIB_12	0,42	0,50	514	0,02	0,06	0,08	0,05	0,20	0,02	0,01	0,04	0,26
13	1406_IGIB_13	0,07	0,05	6,18	0,03	0,05	0,14	0,05	0,13	0,03	0,03	0,05	0,13
14	1406_IGIB_14	2,25	0,89	680	0,04	0,11	0,16	0,05	0,38	0,03	0,01	1,39	0,49
15	1406_IGIB_15	8,58	6,86	3693	0,16	0,56	0,65	0,29	1,19	0,21	0,22	0,34	1,34
16	1406_IGIB_16	0,38	0,34	524	0,06	0,16	0,23	0,07	0,36	0,05	0,05	0,09	0,48
17	1406_IGIB_17	0,05	0,06	363	0,04	0,08	0,13	0,05	0,21	0,06	0,03	0,04	0,23
18	1406_IGIB_18	0,44	0,20	96,3	0,04	0,06	0,13	0,05	0,15	0,05	0,03	0,07	0,21
19	1504_IGIB_21	0,81	0,65	89,2	0,03	0,05	0,07	0,04	0,12	0,01	0,02	6,58	0,15
20	1504_IGIB_22	0,19	0,28	44,3	0,02	0,02	0,06	0,03	0,10	0,01	0,01	0,02	0,12
21	1504_IGIB_23	17,10	17,60	1242	0,08	0,16	0,31	0,27	0,48	18,20	0,23	8,84	0,53
22	1504_IGIB_24	6,32	5,56	1416	0,05	0,16	0,19	0,09	0,63	0,07	0,04	0,10	0,72
23	1504_IGIB_25	4,49	3,80	351	0,23	0,32	0,45	0,30	0,49	0,21	0,29	0,40	0,59
24	1506_IGIB_26	2,48	3,06	347	0,02	0,04	0,07	0,02	0,18	0,01	0,01	70,40	0,16
25	1506_IGIB_27	0,06	0,04	44,2	0,01	0,01	0,03	0,02	0,05	0,01	0,01	0,01	0,07
26	1506_IGIB_28	0,21	0,17	563	0,06	0,09	0,12	0,04	0,24	0,06	0,02	1,48	0,24
27	1506_IGIB_29	0,44	0,71	407	0,03	0,07	0,21	0,05	0,26	0,02	0,03	1,85	0,25
28	1506_IGIB_30	2,76	3,67	603	0,04	0,10	0,17	0,05	0,35	0,04	0,02	0,06	0,35
29	1506_IGIB_31	11,90	9,62	68,0	0,03	0,04	0,16	0,90	0,12	14,10	0,31	0,20	0,11
30	1506_IGIB_32	0,39	0,45	53,7	0,02	0,02	0,05	0,02	0,07	0,05	0,01	0,03	0,08
31	1604_IGIB_33	68,4	36,90	1105	0,04	0,13	0,53	0,59	0,49	82,00	91,50	82,90	0,51
32	1604_IGIB_34	15,60	11,70	475	0,03	0,07	0,33	0,33	0,26	14,60	0,17	26,60	0,23
33	1604_IGIB_35	0,11	0,06	68,7	0,01	0,03	0,06	0,03	0,09	0,02	0,01	17,70	0,08
34	1604_IGIB_36	1,56	0,57	158	0,01	0,02	0,07	0,03	0,12	0,01	0,01	0,03	0,12
35	1604_IGIB_37	3,58	0,28	472	0,03	0,05	0,09	0,04	0,17	0,02	0,01	0,04	0,22

Fig. S1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Per a 2
Per a 3
Per a 4
Per a 5
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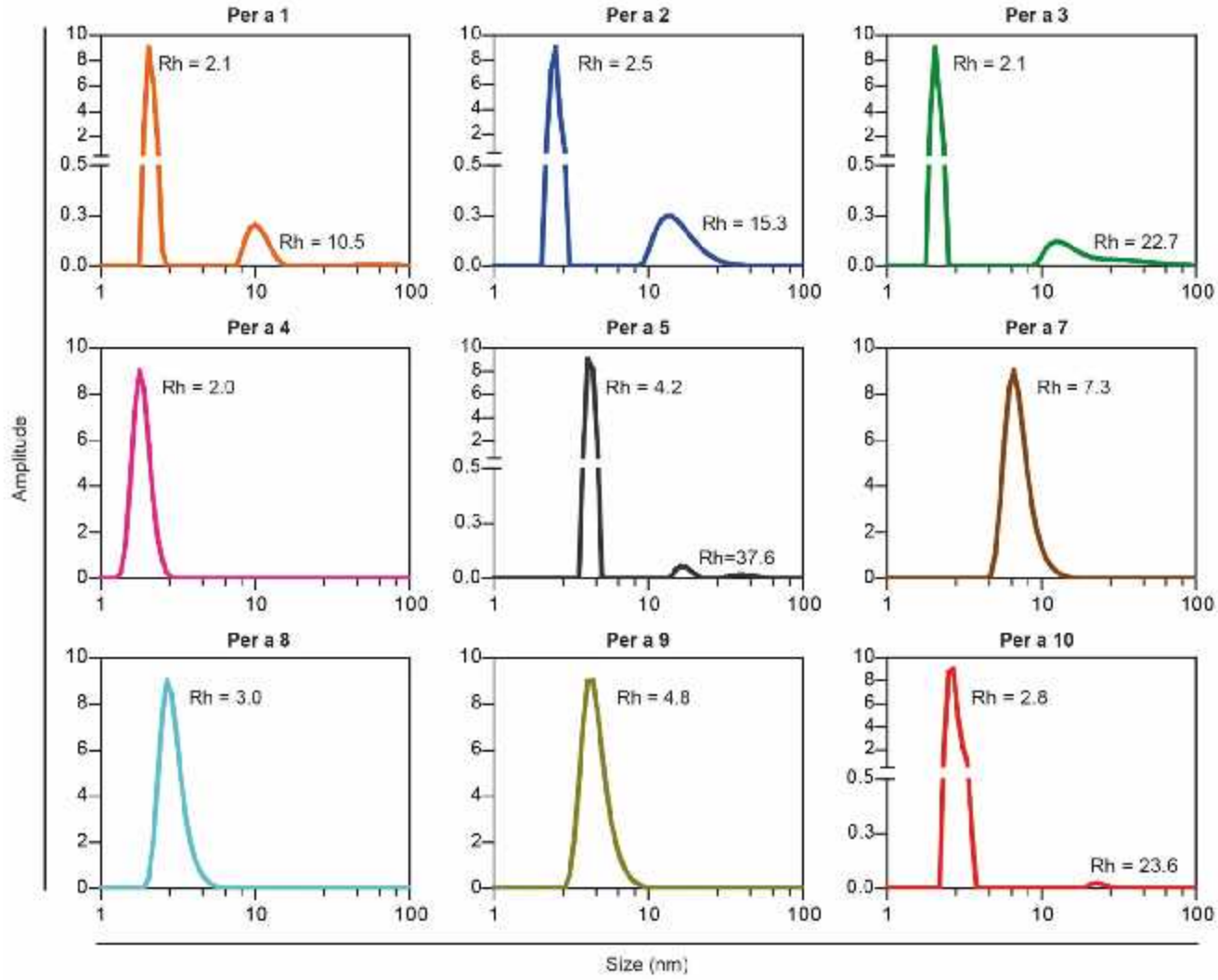
Wavelength (nm)

Fig. S2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Amplitude
Size (nm)

Fig. S3

Per a 4
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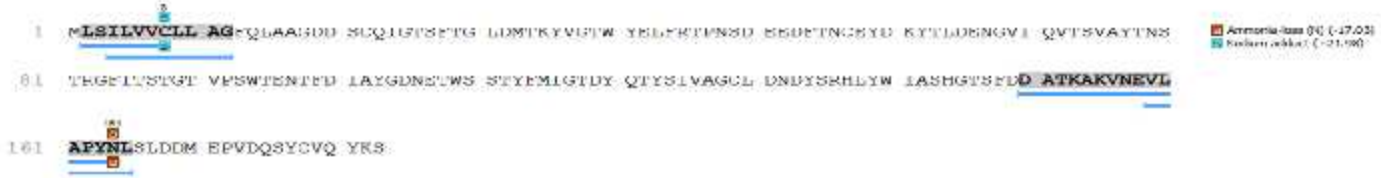

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29%

Per a 8
Extr. 1
34%

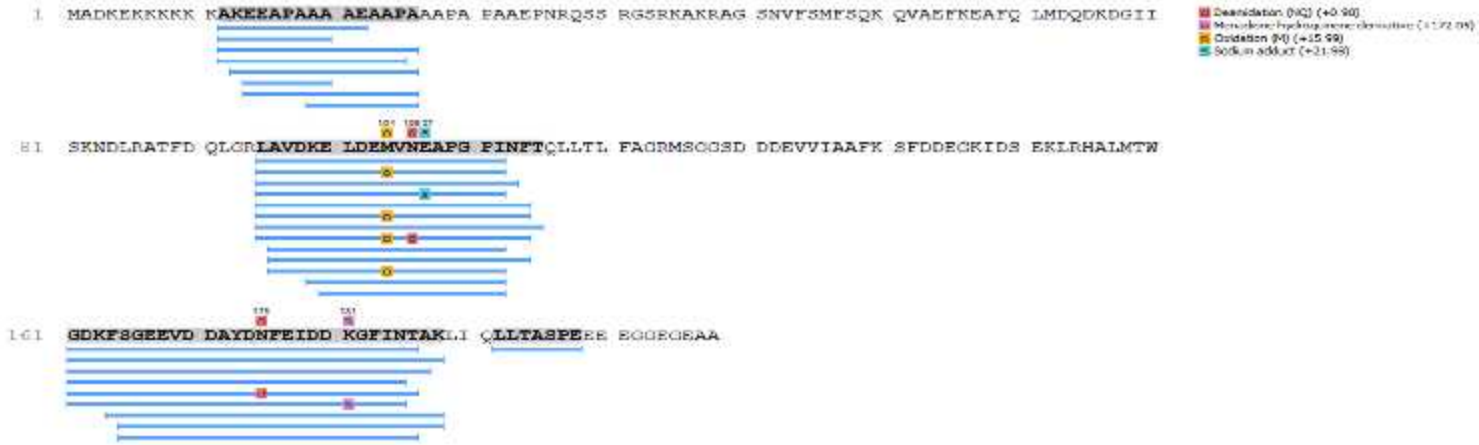
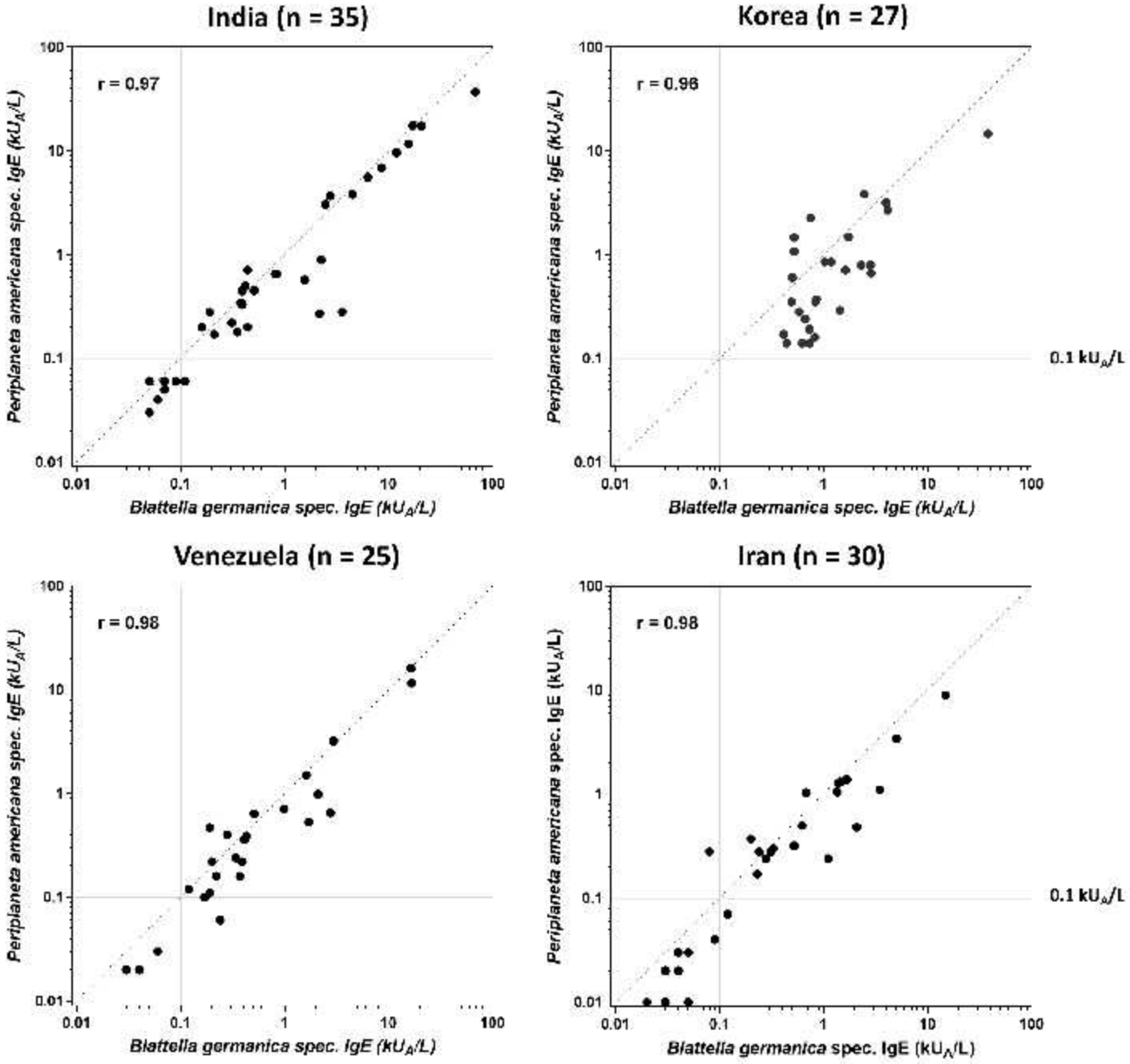


Fig. S4


India (n = 35)
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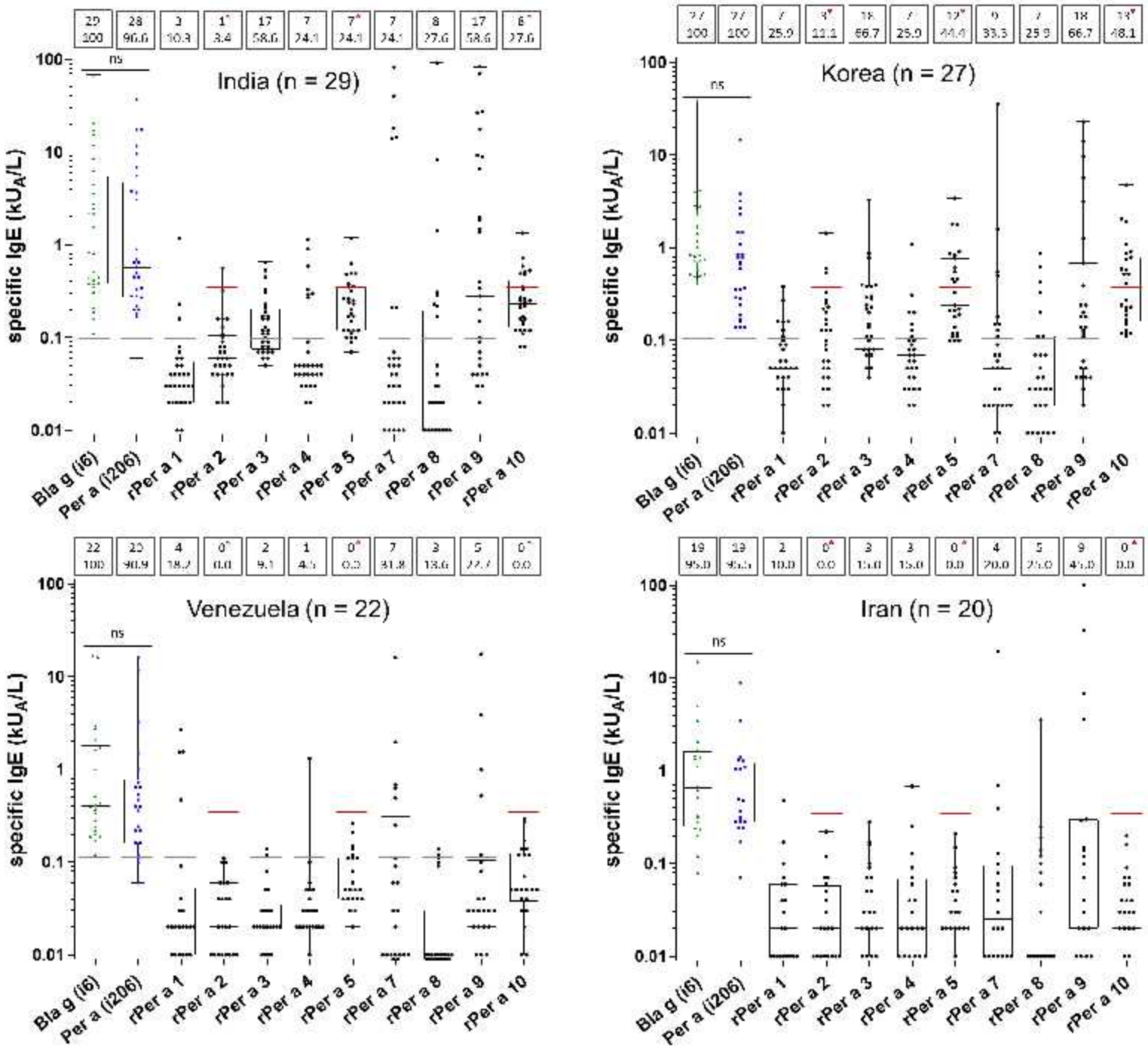
Korea (n = 27)
r = 0.96
0.1 kU$_A$/L
Venezuela (n = 25)
r = 0.98
Iran (n = 30)
r = 0.98
0.1 kU$_A$/L
Periplaneta americana spec. IgE (kU$_A$/L)
Blattella germanica spec. IgE (kU$_A$/L)
0.01
0.1
1
10
100

Fig. S5


India (n = 29)
Korea (n = 27)
Venezuela (n = 22)
Iran (n = 20)
specific IgE (kU$_A$/L)
ns
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Per a (i206)
rPer a 1
rPer a 2
rPer a 3
rPer a 4
rPer a 5
rPer a 7
rPer a 8
rPer a 9
rPer a 10