



Analysis of 10 Environmental Allergen Components of the American Cockroach in Taiwan

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ABSTRACT

Background: Cockroaches are important sources of indoor airborne allergens. The American cockroach (*Periplaneta americana*) is the second leading inhalant allergen causing allergic airway diseases in Taiwan. We previously reported a difference in pathogenicity of different allergen components from American cockroaches.

Objective: To analyze the environmental profile of American cockroach allergen components.

Methods: Polyclonal antibodies were generated to recombinant American cockroach allergens, Per a 1 through Per a 10. The levels of each allergen in (1) whole-body extracts and feces from American cockroaches and in (2) fresh-frozen 6-month-old and 12-month-old dead American cockroaches were evaluated by immunoblotting and quantified. Levels of allergen components from patients' household dust samples were determined by competition enzyme-linked immunosorbent assay.

Results: Per a 1, 2, and 10 proteins were present predominantly in roach feces, whereas other allergen components were found predominantly in roach bodies. There was a time-dependent decrease in total levels of some allergen proteins. Although levels of Per a 4, 5, 6, and 9 significantly decreased to less 20% of the basal level, there was no significant change in levels of Per a 2, 7, and 10 after 1-year decomposition. The most abundant allergen components in 20 dust samples from patients' houses were Per a 9, Per a 10, and Per a 2.

Conclusion: The concentration of 10 American cockroach allergen components differed in the environment. Per a 2 and Per a 10 can be used as markers of long-term environmental cockroach control and Per a 9 as current status of control in patients' houses.

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Introduction

Since the first report linking cockroaches and allergic diseases,¹ cockroaches have been an important source of indoor aeroallergens worldwide. The prevalence of cockroach allergy among people with asthma ranges from 17% to 53% in the United States,² 55% to 79% in Brazil,³ 44% to 60% in Thailand,⁴ and 40% to 57% in Taiwan.^{5,6} The

presence of cockroach allergens in homes is one of the key risk factors predicting allergic sensitization and asthma morbidity in children and adults with asthma. Sensitization to cockroach allergens is associated not only with the development of asthma but also with the severity and mortality of asthma.^{7–9} In addition, the levels of cockroach allergens in settled household dust and indoor air were associated with airway sensitization and asthma morbidity.^{10–14}

Although more than 4000 cockroach species have been identified, only 5 species infest human dwellings.^{15,16} The American cockroach (*Periplaneta americana*) and German cockroach (*Blattella germanica*) are the predominant ones of these species and have been the focus of allergy research. The German cockroach is the dominant domestic cockroach species in temperate areas, whereas

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the American cockroach is more prevalent in tropical and sub-tropical areas. Previous studies identified 12 allergen components (Per a 1 through Per a 12) from the American cockroach.^{15–21}

Sensitization to Per a 2 correlates with a higher severity of airway inflammation compared with the other allergen components, suggesting that the allergen components from the American cockroach have different degrees of pathogenicity.²² The American cockroach is the second leading source of inhalant allergen causing allergic airway disease in Taiwan.⁶ However, no data have described the levels of the different allergen components in the environment. The major aim of this study was to analyze the levels of the 10 most common American cockroach allergens, Per a 1 through 10, in the environment in Taiwan.

Methods

PCR-Based Cloning and Purification of Recombinant Per a 1 Through 10 Proteins

The *P. americana* complementary DNA library was used as a template for polymerase chain reaction (PCR) amplification of the American cockroach allergens Per a 1 through 10. The gene-specific primers for PCR-based cloning were designed with restriction sites for cloning into the pET30 (Novagen, Madison, Wisconsin) and the pQE30 (Qiagen, Hilden, Germany) expression vectors. Sequences of the oligonucleotide primers and the molecular weights of the recombinant allergens are listed in Table 1. Expression vectors pET30 and pQE30, both encoding the histidine tag sequence at the N-terminal ends of the target protein, were amplified in overnight cultures, which were then diluted 1:100 into 200 mL of Luria-Bertani broth containing 25 µg/mL of kanamycin for pET30-BL21(DE3) or 100 µg/mL of ampicillin for pQE30-M15. Protein expression was induced with isopropyl thio-β-D-galactoside at concentrations of 0.1 to 1 mM. The cells were harvested after 16 hours of incubation at 37°C. Recombinant proteins were purified by rapid affinity column chromatography using the histidine tag to bind the target protein to divalent cations (Ni²⁺) immobilized on the histidine-binding metal chelation resin (Novagen). The resulting fusion proteins were recovered by elution with 1 M imidazole according to the manufacturer's instructions (Novagen). The purified proteins were refolded by dialysis against phosphate-buffered saline (PBS). Protein concentration was determined using the Bio-Rad Bradford assay (Bio-Rad, Hercules, California) with bovine

serum albumin as protein standard. Subsequently, the protein preparations were used as immunogenic antigens for production of specific antibodies and as standard allergens in quantitative enzyme-linked immunosorbent assay (ELISA) for measurement of the allergens in house dust samples. The purified recombinant proteins were aliquoted and stored at –70°C until use. Standard curves for quantification of Per a 1 through 10 were generated with serial dilutions from 1.56 to 100 ng/mL of rPer a 1 through 10 as inhibitors.

Generation of Rabbit Polyclonal Antibodies to Recombinant Per a 1 Through 10 Proteins

Antibodies against recombinant proteins were generated in rabbits. Young adult New Zealand white rabbits were injected subcutaneously at 10 to 20 sites on the back with 150-µL aliquots containing a total of 1.0 mg of purified recombinant proteins combined with an equal volume of complete Freund's adjuvant (Sigma, St Louis, Missouri). The rabbits received 2 booster injections of 0.5 mg of antigen mixed with Freund's incomplete adjuvant (Sigma) on days 21 and 42. Starting at week 3, rabbits were bled biweekly, and the antibody reactivity was checked with an indirect ELISA. Three weeks after the last injection, the rabbits were bled by heart puncture. Antisera were purified with an initial 18% sodium sulphate precipitation followed by a 14% sodium sulphate precipitation. The partially purified IgG samples were dialyzed against PBS at 4°C and further purified using recombinant protein G agarose (Invitrogen, Carlsbad, California) affinity chromatography.

Preparation of American Cockroach Whole-Body and Fecal Extracts

American cockroaches were housed in an isolated special terrarium made by our laboratory. The feces were collected from the terrarium. Frozen cockroaches were pulverized in liquid nitrogen and then defatted overnight at 4°C in precooled ether (1:5, wt/vol). After vacuum suction, the residual ether was washed away twice with acetone, and the defatted cockroach extract was lyophilized and stored in –70°C. Defatted whole-body powder was extracted overnight at 4°C in PBS (pH 7.4) (1:20, wt/vol) containing 10 mM phenylmethylsulfonyl fluoride (Sigma). The supernatant was collected by centrifugation at 4°C for 30 minutes at 10,000g and passed through a 0.45-µm filter (Millipore, Bedford, Massachusetts). A 100-mg sample of feces was mixed with 1 mL of PBS and

Table 1
Biochemical Functions and Sequences of Primers Used for the Polymerase Chain Reaction–Based Cloning of *Periplaneta americana* Allergens

Allergen/Accession No.	Biochemical Function	Nucleotide Sequence ^a	Expression Vector	Recombinant Protein Size, kDa
Per a 1.0104/U69261	Midgut microvilli protein homolog	F <u>1GTCGAC CGTTGGTGTTCGATGGTCT</u> R <u>2GCGGCCGCTCAGGGCAGGCCGAACCA</u>	pET30b	37.8
Per a 2.01/GU188391	Inactive aspartic protease	F <u>3GGATCCGATCCAGTCGTCGTTCT</u> R <u>4AAGCTTCTACAGTCTCTTACCGGA</u>	pET30a	48.6
Per a 3.0203/L40821	Insect hemolymph	F <u>5GAATTCGAAATGCCTGCTCTGCC</u> R <u>2GCGGCCGCTCAGTGTGTCACTTCATG</u>	pET30a	51.1
Per a 4/AY792948	Lipocalin	F <u>3GGATCCGGAGATGATTC</u> R <u>4AAGCTTCA AGACTTGTACT</u>	pET30a	30.6
Per a 5/AY92949	Glutathione S-transferase	F <u>3GGATCCATGACCATCGACTTCTAC</u> R <u>4AAGCTTCACTTCTTGGCGAGGTT</u>	pET30a	28.9
Per a 6/AY792950	Troponin C	F <u>3GGATCCATGGATGAAGTCCAGAT</u> R <u>4AAGCTTTATTTCTCCGTCATGAC</u>	pQE30	17.1
Per a 7/Y14854	Tropomyosin	F <u>5GAATTCATGGATGCTATCAAGAAGAAG</u> R <u>2GCGGCCGCTTAGTA ACCAGCAAGTTCGGT</u>	pET30a	42.8
Per a 8/JQ279816	Myosin light chain	F <u>5GAATTCATGGCGGACAAGGAAAAGAAG</u> R <u>2GCGGCCGCTTAAGCGGCTCGCTTCTC</u>	pET30a	27.6
Per a 9/AY563004	Arginine kinase	F <u>3GGATCCATGGTGGACGCCGAGTT</u> R <u>4AAGCTTAGAGCGAGCTCTCCAGCTT</u>	pQE30	41.4
Per a 10/AY792954	Serine protease	F <u>3GGATCCCTAAAGCCAAGCGTCCC</u> R <u>4AAGCTTTAGTTGACTCCAGTCTGTTT</u>	pQE30	24.5

^aRestriction sites are in italics and priming regions are underlined. F indicates forward; R, reverse; ¹, *Sall*; ², *NotI*; ³, *BamHI*; ⁴, *HindIII*; and ⁵, *EcoRI*.

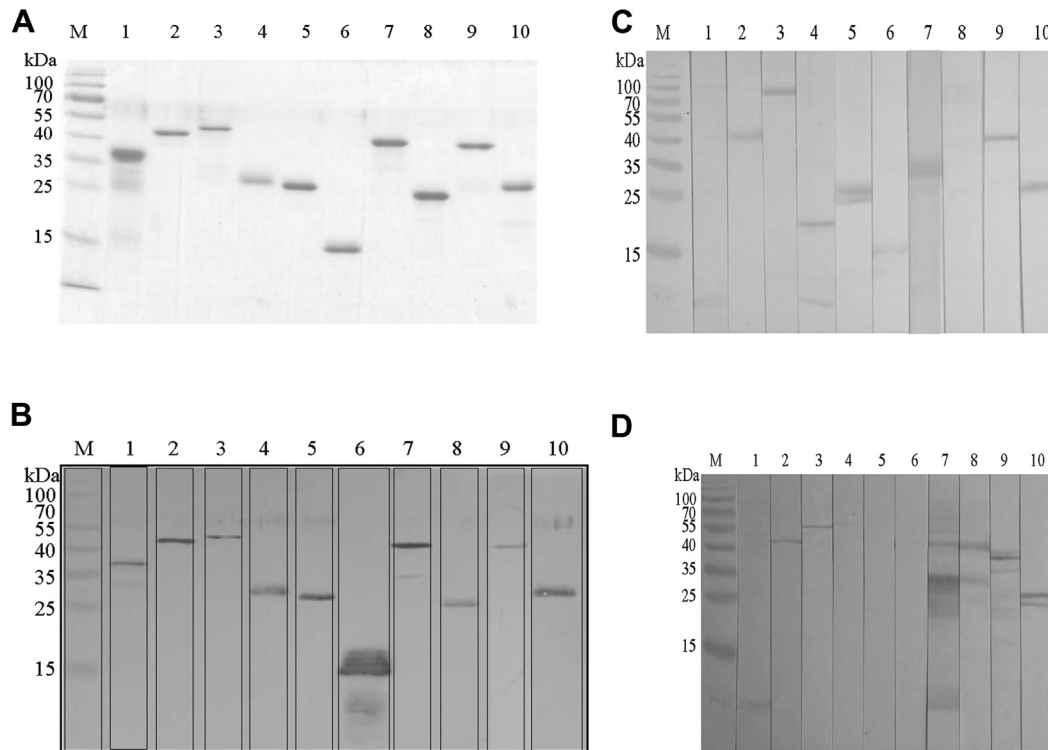


Figure 1. A, Coomassie Blue–stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis of 10 purified recombinant cockroach allergens expressed in *Escherichia coli*. Lanes 1 through 10, recombinant Per a 1 through 10, respectively. Immunoblotting of recombinant Per a 1 through 10 (B), whole-body extracts from American cockroaches (C), and German cockroaches (D) (Greer Companies) with individual rabbit anti–recombinant Per a 1 through 10 specific antibodies (lanes 1–10, respectively). We produced 10 polyclonal antibodies against recombinant Per a 1 through 10 and demonstrated the specific recognition for both the recombinant and natural allergens.

extracted at 4°C overnight. The preparation was then centrifuged at 10,000g at 4°C for 30 minutes. The protein contents of the American cockroach and fecal extracts were measured by Bradford assay (Bio-Rad). The extracts were stored at –70°C until use. For decomposition study of dead roaches, 10 adult fresh-frozen cockroaches were weighed and transferred to 50-mL centrifuge tubes covered with gauze and exposed in household condition for 6- and 12-month intervals. After finishing the decomposition period, the tubes were locked and stored in the freezer until used.

SDS-PAGE and Immunoblotting for Antibody Specificity and Semiquantification of Per a 1 Through 10 in American Cockroach and Feces

The purity of *Escherichia coli*–expressed Per a 1 through 10 recombinant proteins and the specificity of laboratory-prepared Per a 1 through 10 antisera were checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Briefly, 0.1 to 1 µg of each recombinant Per a 1 through 10 and 10 µg of American cockroach were separated on 4% to 12% SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue R-250. For immunoblotting, the gels were transferred to nitrocellulose membranes and blocked with 2% bovine serum albumin for 1 hour. The membranes were then probed with individual rabbit anti–Per a 1 through 10 antibodies (0.5 µg/mL) for 1 hour at room temperature. The membranes were washed with PBS with Tween 20, incubated with peroxidase-labeled goat anti-rabbit IgG antibody (10,000-fold dilution; Millipore) for 1 hour, and visualized using 3-amino-9-ethylcarbazole as an enzyme substrate. Density of the protein bands was quantified using Gel-Pro image analysis software version 3.1 (Media Cybernetics, Rockville, Maryland).

House Dust Collection and Extraction of the Dust Samples

Dust samples were collected from the homes of cockroach-allergic patients with asthma who visited the Allergy Special Clinic of the hospital. Allergy to cockroach in patients with asthma was determined based on a positive serum level of allergen specific IgE to cockroach (>0.35 kU/L) by ImmunoCAP (Thermo Fisher Scientific Inc, Phadia AB, Uppsala, Sweden). Individuals were instructed to bring a sample of dust taken from their vacuum cleaner. The collected dust was from a variety of types of vacuum cleaners and represented a general collection from the entire house. Individuals who submitted dust samples were asked to complete a questionnaire concerning the source of the dust and the general environment. On its arrival, dust samples were immediately frozen until processed for analysis. Before extraction, each dust sample was sieved through a 425-µm mesh and weighed. A 100-mg sample of the fine dust was suspended in 1 mL of PBS containing 0.05% Tween 20 and mixed overnight on a rocker platform at 4°C. The preparation was then centrifuged at 5,000g for 10 minutes. The supernatant was stored at –20°C until used in a quantitative ELISA for determining the levels of Per a 1 through 10.

This project was approved by the institutional review board of Taichung Veterans General Hospital. All study patients provided signed informed consent forms.

Competition ELISA for Quantification of Per a 1 Through 10 in Dust Samples

Optimal concentrations of antigen and conjugate were determined by checkerboard titration. First, inhibition plates were prepared by blocking with 3% nonfat milk and PBS overnight at room temperature. After washing with PBS with Tween 20, a total of 3 two-fold dilutions of each test extract (100 µL per well) and 5 two-fold dilutions of recombinant Per a 1 through 10 proteins were

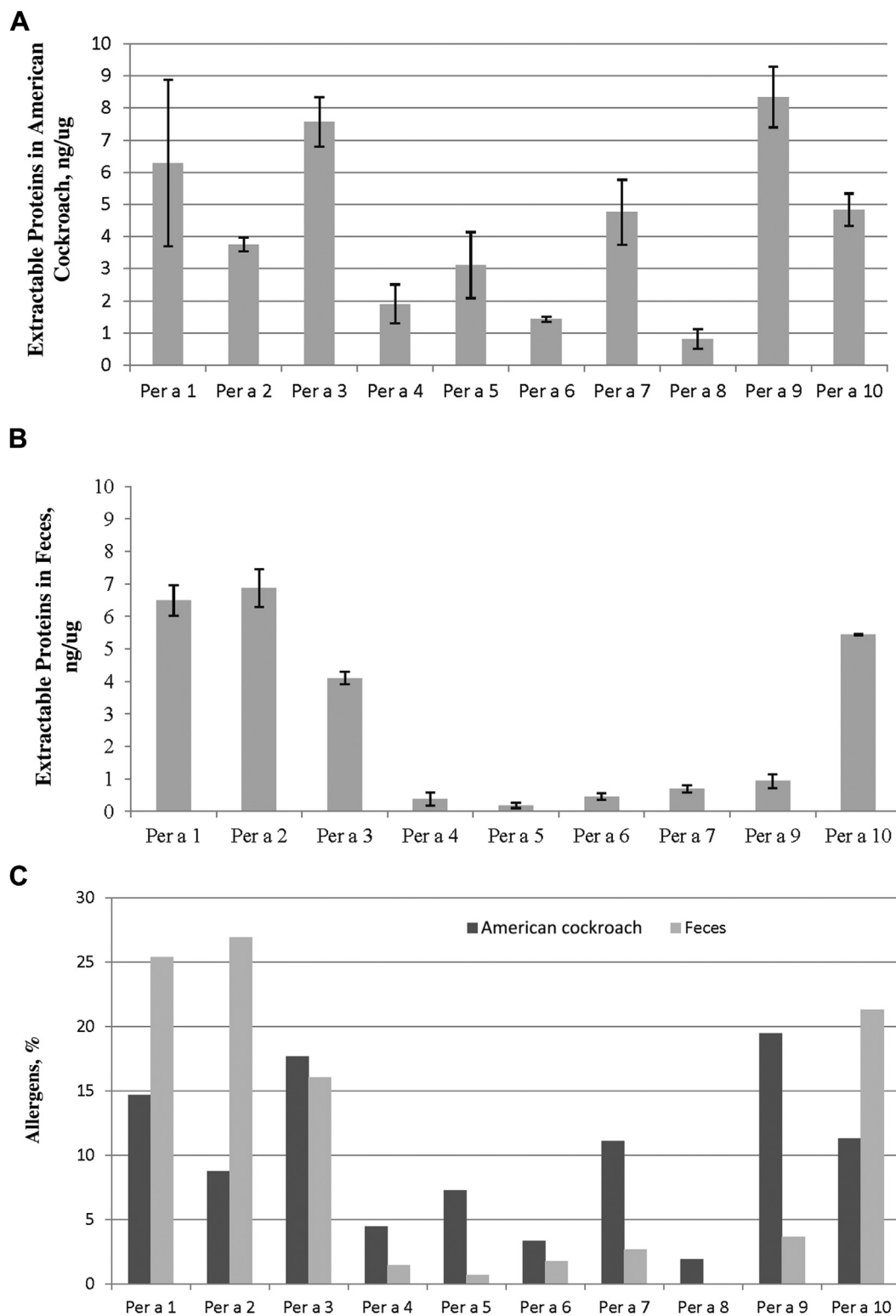


Figure 2. Allergen levels of whole-body extracts (A) and feces (B) from American cockroaches determined by immunoblotting. Semiquantitative analysis of the specific bands was performed with Gel-Pro image analysis software. C, Individual allergen levels by percentages of total allergens in American cockroach and feces, respectively. The results revealed that the allergen patterns in the whole-body extracts were apparently different from that in the feces, which matched their biological nature.

made in duplicate wells. Rabbit anti-recombinant Per a 1 through 10 antibodies were added to each sample dilution (100 μ L per well). The plates were incubated for 2 hours at 37°C.

In addition, microtiter plates that contained the solid-phase antigen were prepared by coating wells with recombinant

Per a 1 through 10 (5 ng per well) in a carbonate buffer (pH 9.6). After incubation for 2 hours at 37°C, the plates were blocked for 2 hours with 2% bovine serum albumin and incubated with antibody for 2 hours. The inhibited antiserum was then transferred to 96-well assay plates that contained the solid-phase antigen and incubated

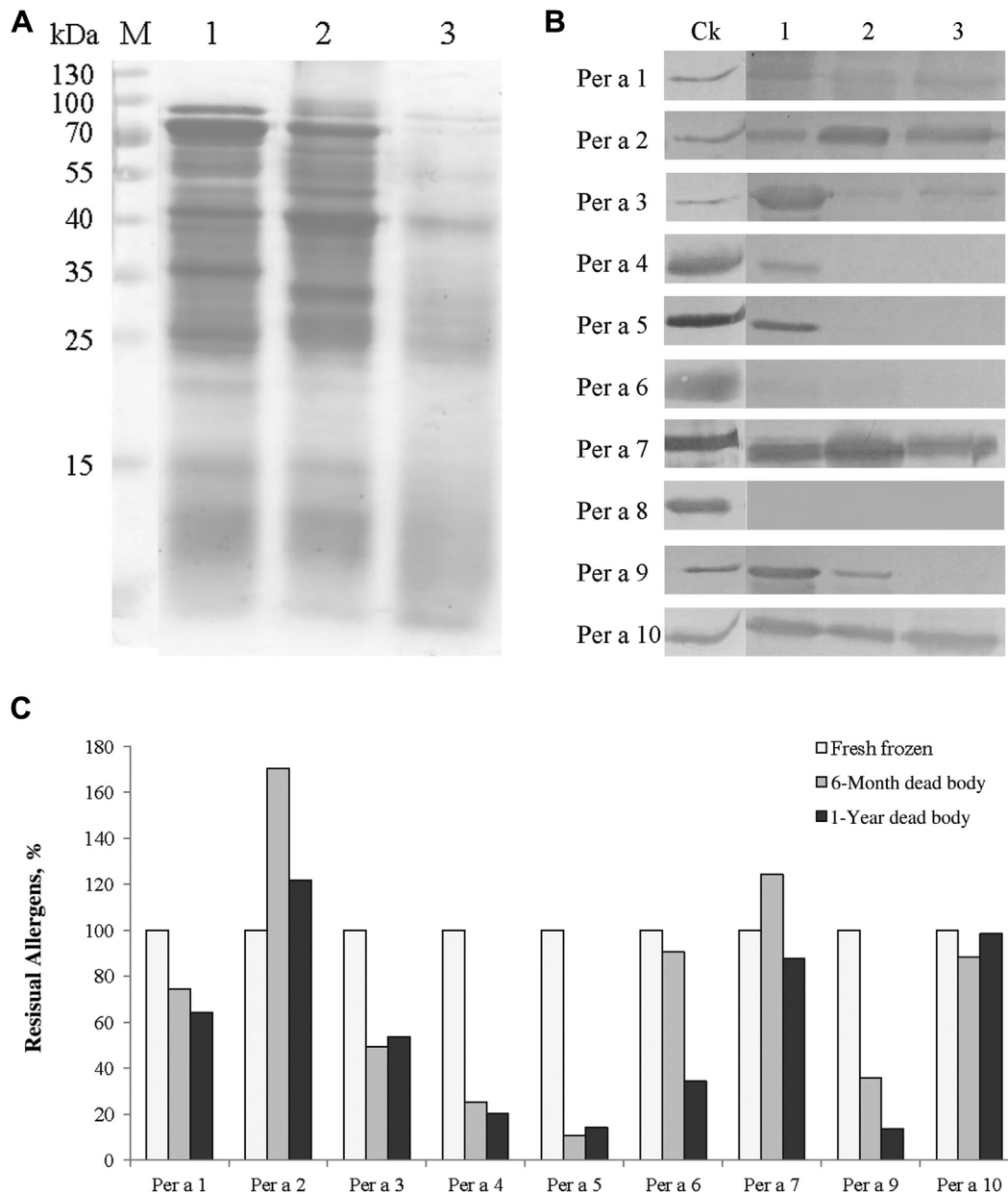


Figure 3. Coomassie Blue–stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (A) and immunoblotting (B) of American cockroach with rabbit anti–Per a 1 through 10 polyclonal antibodies. C, Percentage of residual Per a 1 through 10 proteins in American cockroach from panel B. Lane 1, fresh-frozen American cockroach; lane 2, American cockroach from 6-month dead body; lane 3, American cockroach from 1-year dead body. Lane Ck represents individual recombinant Per a 1 through 10 (100 ng per band) as standards of semiquantification. The results revealed that natural proteins of Per a 2, 7, and 10 are more resistant to decomposition than other cockroach allergens.

for 2 hours at room temperature to allow the remaining unbound antibodies to bind with the solid-phase antigen. After washing, a 1/10,000 dilution of peroxidase-labeled goat anti-rabbit IgG was added and incubated for 1 hour at room temperature, and a colored reaction was developed by the addition of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (55 μ g/mL in 0.1 M sodium citrate buffer, pH 4.2, containing 0.03% of H_2O_2). The results were read at 415 nm on a Sunrise Reader (Tecan Austria GmbH, Grödig, Austria). The concentration of Per a 1 through 10 in the test samples were determined by comparing the reference standards.

Statistical Analyses

Exposure and individual allergen associations were assessed by categorizing the dust allergens into 3 groups (below detectable limits and median and high levels in nanograms per gram of dust)

and performing analysis of variance for trends (SPSS Inc, Chicago, Illinois).

Results

SDS-PAGE of Recombinant Allergens and Immunoblotting of American Cockroach With Rabbit Anti–Per a 1 Through 10 Antibodies

Complementary DNA fragments encoding the Per a 1 through 10 genes were inserted into the pET30 or pQE30 vectors and expressed in *Escherichia coli* as recombinant proteins. The 10 recombinant proteins were purified using affinity chromatography and subjected to SDS-PAGE. All 10 recombinant proteins migrated as a single band and had more than 99% purity by Coomassie Blue staining (Fig 1A). Polyclonal antibodies against the purified recombinant Per a 1

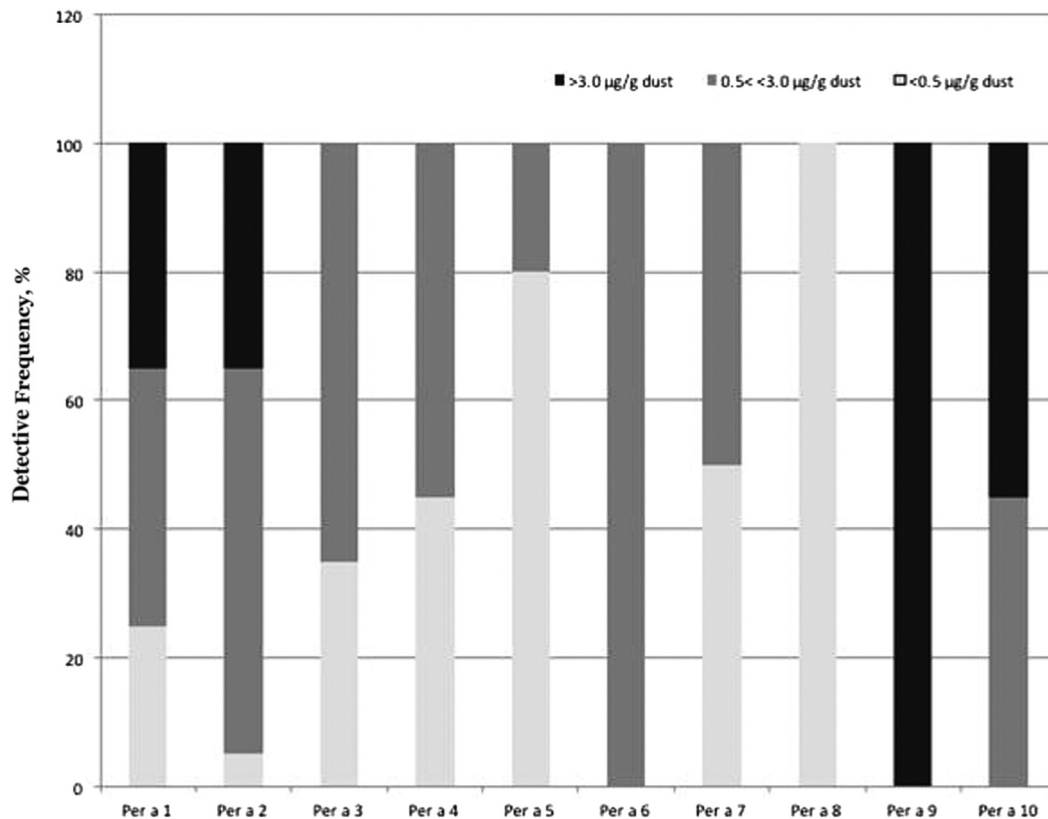


Figure 4. Determination of Per a 1 through 10 concentrations in 20 dust samples by inhibition enzyme-linked immunosorbent assay. The limit of detection of the assay was 0.011 µg/g of dust for each individual allergen. Our preliminary data suggested that Per a 9, Per a 10, and Per a 2 are the top 3 most abundant cockroach allergen components in the Taiwanese house dusts.

through 10 were generated in New Zealand white rabbits, and their specific reactivities were determined by immunoblotting (Fig 1B). Furthermore, the binding specificities of rabbit anti–recombinant Per a 1 through 10 antibodies to American cockroach and whole-body extracts from German cockroaches were also confirmed (Fig 1C and D). Interestingly, native Per a 8 was very rare and only detected from the extraction of enriched head and thorax muscle (data not shown).

Allergen Levels in American Cockroach and Feces by Immunoblotting

The levels of the 10 American cockroach allergens were evaluated by Gel-Pro image analysis software from immunoblots. We determined that approximately 4.28% and 2.55% of extractable proteins from American cockroach and feces had detectable levels of cockroach allergens, respectively. The individual allergen levels in American cockroach and feces are shown in Figure 2A and B. Immunoblotting was used to show that there was a difference in the levels of components in American cockroach and that in feces. American cockroach had higher levels of Per a 1, 3, and 9 allergens (>15% of total allergen contents) compared with other allergen components, whereas feces had higher levels of Per a 1, 2, and 10 (>20% of total allergen contents) (Fig 2C).

American cockroach from fresh-frozen cockroaches and cockroaches dead for 6 months and 1 year were prepared to evaluate the stability of allergens in adult cockroaches under household conditions. Immunoblotting was used to determine the content of allergens Per a 1 through 10 in these extracts (Fig 3). Our data indicate a time-dependent decrease in the amount of total proteins (Fig 3A, lanes 1–3). There was a significant decrease in the concentrations of Per a 4, 5, 6, and 9 (<20% of the basal level after 1 year

of decomposition). However, there was no significant change in the concentrations of Per a 2, 7, and 10 after 1 year of decomposition (>80% of basal level from fresh-frozen American cockroach) (Fig 3B and C).

Measurement of Cockroach Allergens in Patients' Household Dusts

Twenty dust samples from patients' houses were collected, and competition ELISA was used to determine the levels of the 10 allergen components (Fig 4). The most commonly found allergen components in the 20 dust samples were Per a 9, Per a 10, and Per a 2. All the dust samples had high levels of Per a 9 (>3.0 µg/g dust), and 95% of the samples had moderate to high levels of Per a 10 and Per a 2. Per a 8 was found at very low levels in all samples.

Discussion

Like mite allergens, there was a strong association between the degree of cockroach sensitization and increasing domestic concentrations of cockroach allergens in allergic children.^{23–25} These studies suggested that although complete removal of cockroach allergens from the infested environment is difficult, it may be helpful to reduce cockroach allergens to below clinical relevant thresholds.^{26–29} It is known that in addition to the bodies of roaches, the cast skins, egg shells, and feces could also be sources of allergens. There is increasing knowledge of the biochemical functions of the different allergen components. Our present study found the presence of Per a 1, 2, and 10 predominantly in roach feces, whereas Per a 4, 5, 6, 7, and 8 were predominantly found in roach bodies. We also found that Per a 6, 7, and 8 proteins were part of the muscle components. Per a 4 is calyxin, a fatty acid-binding protein,

and Per a 5 is a glutathione S-transferase.³⁰ The biological nature of these proteins explained why they are predominantly detected in cockroach bodies but not the feces.

It is possible that allergen components that exist predominantly in roach feces or are more resistant to decomposition in the environment reach a much higher concentration over time compared with allergens that exist in the roach bodies or are vulnerable in the environment. We previously reported that Per a 2 is predominantly localized in the hindgut and could be excreted into the feces.¹⁷ In this study, we found that Per a 2, 7, and 10 were the most robust roach allergens and that more than 80% of these proteins remained intact after 1 year of decomposition. It was previously found that under conditions of suitable temperature and humidity, Bla g 2, the major group 2 German cockroach allergen,³¹ could exist in an apartment with a half-life of 406 days.³² Our data indicate that there was only a 20% decrease in Per a 2 levels after 365 days of decomposition. Per a 2 concentrations exceeded 0.5 µg/g, which is the lowest clinical relevant threshold, in the dust samples from 95% of the samples that we tested.²⁹ However, the sample size of the dust samples collected was small in current study. It is important to use larger sample sizes to confirm whether Per a 9, Per a 10, and Per a 2 are the most abundant allergen components in Taiwanese patients' houses. It will also be interesting to study whether there are seasonal differences as well. In addition, as Per a 7 extensively cross-reacts with dust mites and midges, as shown in eFigure 1, it is possible that environmental samples could be giving an elevated reading for Per a 7 because of potential sample contamination with dust mites, midges, or other insects in the environments.

Per a 9 has been reported to be an important cockroach allergen correlated with disease exacerbation in Thailand.³³ It is the most abundant allergen detected in the dust samples in our study. All the dust samples that we tested exhibited Per a 9 levels above 3 µg/g dust. However, Per a 9 protein decomposes rapidly in the environment, and existence of Per a 9 in the environment may indicate a current American cockroach infestation and inadequate environmental cockroach control. Per a 9 can therefore be used as a marker of current environmental cockroach control. In contrast, Per a 2 and Per a 10 are relatively stable in the environment and are therefore good candidate markers for long-term successful environmental cockroach control.

In conclusion, there are differences in the concentrations of 10 American cockroach allergen components in the environment. Our data suggested that Per a 2 and Per a 10 levels could be used as markers of long-term environmental cockroach control, whereas Per a 9 levels could be an indicator of the current status of environmental cockroach control in patients' houses.

Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2016.09.432>.

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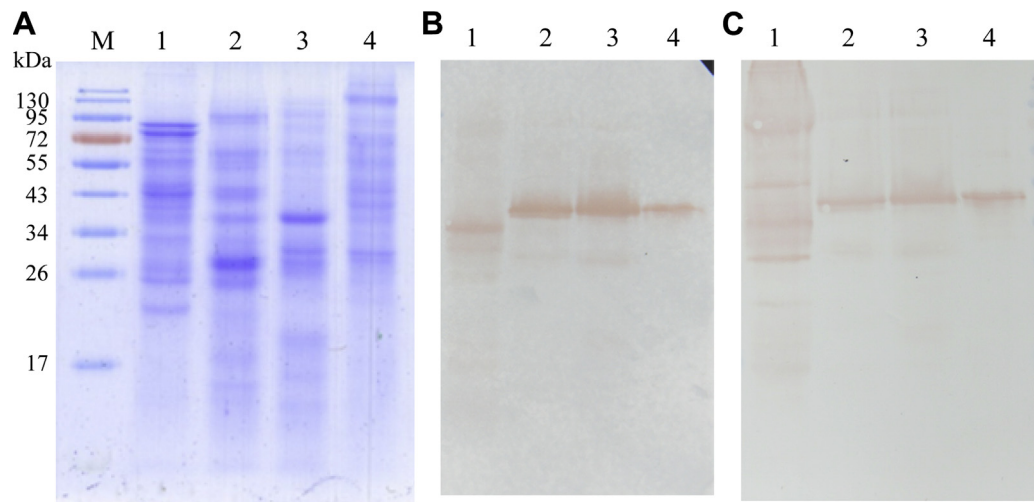


Figure 1. Cross-reactivity of polyclonal antibodies raised against recombinant Per a 7 or recombinant Per a 1 through 10 to extracts from 4 arthropods. Coomassie Blue–stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (A), immunoblotting with rabbit anti–recombinant Per a 7 antibody (B), and immunoblotting with mixture of rabbit anti–recombinant Per a 1 through 10 antibodies (C). Lane M indicates prestained protein marker; lane 1, extract from Per a (American cockroach); lane 2, Der p (mite); lane 3, Der f (mite); and lane 4, for t (biting midge). The results indicate that anti–recombinant Per a 7 antibody cross-react with house dust mites and biting midges, very likely because of the extensive existence of tropomyosin within these arthropods.