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# Seasonal Levels of the Major American Cockroach Allergen Per a 9 (Arginine Kinase) in Bangkok and Their Relevance for Disease Severity

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**SUMMARY** Monitoring the levels of cockroach (CR) allergen in the environment has medical relevance as a clear dose response relationship between CR allergen exposure, sensitization and hospitalization has been reported. In this study, a cross-sectional survey of the levels of a major American cockroach (*Periplaneta americana*) allergen, *i.e.* Per a 9 (arginine kinase) in dust samples collected in various seasons throughout the year 2007 from 76 houses of CR allergic Thai patients in the Bangkok metropolitan area were determined. A monoclonal antibody-polyclonal antibody (MAb-PAb) based-sandwich ELISA was used. The MAb was specific to Per a 9 and the PAb was raised in a rabbit against the crude extract of *P. americana*. The detection limit of the assay was 122 pg of the allergen or 0.024 µg per gram of fine dust powder. The concentrations of Per a 9 were found to be highest during the winter months and lowest in summer. The levels of this CR allergen had a direct correlation with disease exacerbation; *i.e.* the majority of the CR allergic patients had their most severe clinical manifestations during winter. Moreover, the CR allergen levels were found to be higher in wood based-houses than in concrete houses.

Allergies and asthma caused by environmental allergens, both outdoor and indoor allergens, are currently growing global health problems.[1] A common trigger for both maladies are cockroach (CR) allergens derived from CR infestation of the houses.[2] Individuals who live in CR infested houses especially infants and young children are exposed and prone to become sensitized by inhalation of the potent CR allergens. This occurs not only in the inner city or urban areas but also in suburbs, small towns and the countryside everywhere. Sensitized subjects produce specific IgE to CR allergens which are fixed to the surface of mast cells and basophils. These individuals are at high risk of affliction especially asthmatic attacks upon re-exposure to the same insect allergens.[3] The morbidity of CR allergy is more severe and prolonged than the clinical manifestations caused by other indoor allergens.[4,5] The CR allergic attacks are not seasonal and may occur at any time throughout the year indicating that the CR allergens are perennially sustained in the environ-

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ment, *i.e.* the homes of the sensitized subjects.[6] CR allergens are highly mobile and can be found throughout the insect infested house but in different levels in the kitchen, living room, and bedroom, in falling order.[7] In general, the kitchen levels were the best indicator of the magnitude of the CR infestations, but exposures to the allergens in the bedrooms and living rooms have been found to be more relevant in causing sensitization and resurgence of morbidity.[8]

In Thailand, the predominant domiciliary CR species is the American CR, *Periplaneta americana*.[7] These insects produce a variety of allergens.[9] CR allergy in Thailand, like elsewhere, is increasing and the prevalence ranges from 44-61%.[10,11] The major CR allergens that bound to serum IgE of all CR allergic Thais were Per a 1 and Per a 9.[12,13] The levels of Per a 1 in homes of CR allergic Thais were determined in 2004 by a monoclonal antibody (specific to Per a 1)-polyclonal antibody (to CR extract) (MAb-PAb) based sandwich ELISA.[7] In this study, we determined seasonal variations of the levels of the other major American cockroach allergen, Per a 9 (*P. americana* arginine kinase), in the homes of Thai patients with CR allergy. This information should be useful not only for disease prevention/intervention of CR allergic subjects but also for reducing exposure and sensitization of naïve subjects.

## MATERIALS AND METHODS

### Houses, dust sampling, and extraction of the dust samples

Dust samples were collected from 76 Bangkok dwellings of CR allergic patients of Siriraj Hospital who developed clinical symptoms and were positive by skin test to CR extract. Open-and closed-ended questionnaires were used to obtain the family history of the patients' allergic diseases as well as other information including materials used for constructing the houses and the house characteristics, pets, insecticide usage, etc. Dust collections from each dwelling were done three times in different seasons throughout the year 2007: March-April for summer, July-August for the rainy season, and November-December for winter. Each dust collection was performed in the bedrooms, living rooms, and kitchens. However, some families lived in one room apartments or huts; thus, in this case, dust samples were collected from the indicated bedding/sleeping, television watching and cooking and eating areas instead of separate bedrooms, living rooms and kitchens, respectively. Every dust collection was always operated by the same person who collected each sample over an area of one square meter for two minutes. The sample was strained through a fine strainer and 0.1 g of the fine dust powder was mixed with 2 ml phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST) and blended well. The preparation was then centrifuged at 5,000 x *g* at 25ºC for 10 minutes. The supernatant was collected and kept at -20 ºC until use in a sandwich ELISA for determining the Per a 9 level.

### Murine monoclonal antibody (MAb) to Per a 9 (arginine kinase) of *P. americana*

The hybridoma clone 38G6 which secreted MAb of IgG1 isotype to Per a 9 (MAb38G6)[13,14] was grown in serum-free medium (Gibco, Invitrogen Corp, Grand Island, NY, USA). The spent medium was collected and the indirect ELISA titer was determined against crude *P. americana* extract.

### Rabbit polyclonal antibodies (PAb) to *P. americana* extract

PAb to crude American CR produced previously was used.[14] The preparation originated from the immune serum of a New Zealand White rabbit which had been immunized with 5 intramuscular injections of American CR extract at 14 day intervals. The first dose consisted of 1 mg of the extract mixed with complete Freund's adjuvant and each subsequent dose consisted of 0.5 mg of the same antigen mixed with incomplete Freund's adjuvant. The rabbit was bled 14 days after the last booster dose. The total IgG fraction was obtained from the immune serum by ammonium sulfate precipitation and protein-G affinity column chromatography. The protein content of the polyclonal IgG was determined[15] and adjusted to 1 mg per ml in PBS.

### Preparation of native Per a 9 (arginine kinase) of *P. americana*

Native arginine kinase was prepared from crude American CR extract by affinity chromatogra-

phy as previously described.[13] Briefly, purified MAb38G6 were coupled to Sepharose CL4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instruction. The affinity gel was mixed with the crude CR extract at 4ºC overnight, the gel was washed several times with PBS to remove unbound proteins, packed into a column and the bound arginine kinase was eluted out with 0.1 M glycine-HCl buffer, pH 3.0. One ml fractions were collected into tubes containing two drops of 1 M Tris-HCl pH 8.0. The contents in the tubes showing an optical density at an absorbance of 280 nm ($A_{280nm}$) were pooled and dialyzed against distilled water at 4ºC overnight. The preparation was used as standard allergen in the sandwich ELISA for quantification of the allergen in the house dust samples.

### Sandwich ELISA for quantification of *P. americana* Per a 9 (arginine kinase) in dust samples

The sandwich ELISA for quantification of Per a 9 was performed as previously described.[13] Briefly, 4 µg of MAb38G6 in 100 µl of carbonate-bicarbonate buffer were used to coat each well of an ELISA plate. After incubation the wells were washed with PBST, blocked with 1% BSA in PBST, filled with 100 µl of dust sample each (5 mg of fine dust powder) and incubated at 37ºC for one hour. After washing all wells with PBST, PAb (100 µl) was added to each well and the plate was kept at 37ºC for one hour. Excess PAb was washed off and 1:2,000 goat anti-rabbit IgG-HRP conjugate was added to each well (100 µl per well), incubated at 37ºC for one hour and washed as above. Thereafter, peroxidase substrate was added appropriately to each well for color development and the plate was kept for 30 minutes. The enzymatic reaction was stopped by adding 50 µl of 4N $H_2SO_4$ to each well. The OD of the content in each well was measured at $A_{492nm}$ against a blank (a well to which PBS was added instead of the dust samples) with an ELISA reader (Multiscan *EX*; Lab systems, Helsinki, Finland). A series of different amounts of native Per a 9 (arginine kinase) was included in the same plate as allergen standard. The amounts of Per a 9 in the test dust samples were determined from a standard curve constructed by plotting the amounts of standard Per a 9 content with the read-out ELISA OD. The lowest amount of the purified Per a 9 that could be detected by the sandwich ELISA was 0.122 pg of pure Per a 9 which was equivalent to 0.024 µg per gram of fine dust.

### Statistical methods

The Kruskal-Wallis and Mann-Whitney U-Wilcoxon Rank Sum W tests (two tailed) of the computer programs Minitab and SPSS were used for comparison of the medians and ranges of the allergen contents in the dust samples. The concentrations of the allergen at a 95% confidence interval (CI) were used in the comparisons.

## RESULTS

Characteristics and construction materials of the dwellings of the CR allergic patients are shown in Table 1. The patients' houses could be arbitrarily classified into 5 types, *i.e.* single/isolated houses (n = 45), mercantile settings (n = 4), urban slum huts (n = 2), town/terrace houses (n = 9) and high rise apartments/condominiums (n = 6). The majority (59.2 %)

**Table 1** Characteristics and construction materials of the dwellings of the CR allergic patients

| Characteristic of the dwellings | No. of dwellings (%) | Construction material(s) | | |
|---|---|---|---|---|
| | | Wood | Concrete and wood | Concrete |
| Single (isolated) house | 45 (59.2) | 5 (11.1) | 18 (40.0) | 22 (48.9%) |
| Mercantile setting | 14 (18.4) | - | 4 (28.6) | 10 (71.4%) |
| Urban slum hut (one room) | 2 (2.6) | 2 (100) | - | - |
| Town/terrace house | 9 (11.8) | - | 2 (22.2) | 7 (77.8%) |
| High-rise apartment | 6 (7.9) | - | - | 6 (100%) |
| Total | 76 (100%) | 7 (9.2%) | 24 (31.6) | 45 (59.2) |

of the houses was of the single/isolated house type and there were only 2 slum huts (2.6%) included in the study. As for materials used for constructing the dwellings, 59.2% were concrete, 31.6% were a combination of concrete and wood and 9.2 % were wood only.

As shown in Table 2 the ranges of the Per a 9 levels in various rooms/areas of the patients' houses varied greatly from undetectable (9.8-15.4% of the living rooms/television watching areas, 2.9-18.5% of bedrooms/sleeping areas and 3.0-16.4% of kitchens/cooking and eating areas) to as high as 588 μg per gram of fine dust powder. Because of such great variability, the median values are presented in this communication instead of the means ± standard deviations. Moreover, only detectable allergen levels were included in the subsequent analysis. From Table 3 it can be seen that the slum huts had significantly higher median Per a 9 allergen levels than the other types of houses, except for the summer period. The levels of the allergen in all room types were significantly higher in winter than in summer ($p < 0.05$). The levels in the living rooms/television watching areas were also higher in winter than in the rainy season ($p < 0.05$) (Table 4). The allergen levels in the bedrooms/sleeping areas and kitchens/cooking and eating areas in winter and the rainy season were not different but were significantly higher than the levels in the respective areas in summer ($p < 0.05$) (Table 4).

The Per a 9 levels with regard to the materials used for constructing the houses are shown in Table 5. The concrete houses had significantly lower allergen levels in summer and the rainy season than the houses made of wood only or wood and concrete in combination ($p < 0.05$). For all houses the Per a 9 levels were lowest in summer. In houses made of wood only the allergen levels were markedly higher

**Table 2** Numbers of samples with undetectable and detectable levels of Per a 9 according to seasons and room types

| Season | Room type | | | | | |
|---|---|---|---|---|---|---|
| | Living room/television watching area | | Bedroom/sleeping area | | Kitchen/cooking and eating area | |
| | Undetectable | Detectable | Undetectable | Detectable | Undetectable | Detectable |
| **Summer** | 5/51* (9.8%) | 46/51 (90.2%) | 5/55 (9.1%) | 50/55 (90.9%) | 9/55 (16.4%) | 46/55 (83.6%) |
| **Rainy season** | 8/52 (15.4%) | 44/52 (84.6%) | 10/54 (18.5%) | 44/54 (81.5%) | 7/54 (13.0%) | 47/54 (87.0%) |
| **Winter** | 8/67 (11.9%) | 59/67 (88.1%) | 2/68 (2.9%) | 66/68 (97.1%) | 2/67 (3.0%) | 65/67 (97%) |

*Number of samples/Total number of samples collected

**Table 3** Medians (ranges) of Per a 9 levels in the different types of houses during different seasons

| Type of houses | Median (range) of Per a 9 in μg/g of dust during the different seasons | | | |
|---|---|---|---|---|
| | All year round | Summer | Rainy season | Winter |
| **Single (isolated) house** | 3.97[a] (0.312-588.67) | 2.40[a] (0.312-229.85) | 7.40[a] (0.312-524.14) | 7.25[a] (0.312-588.67) |
| **Mercantile setting** | 8.08[a] (0.312-313.67) | 4.00[a] (0.312-277.00) | 14.45[a] (0.37-115.38) | 11.31[a] (0.33-313.67) |
| **Urban slum hut** | 20.64[b] (0.33-181.29) | 0.78[a] (0.33-175.75) | 50.25[c] (1.78-50.25) | 40.69[c] (0.37-181.29) |
| **Town/terrace house** | 1.50[a] (0.312-127.27) | 1.16[a] (0.70-1.63) | 1.16[a] (0.74-1.33) | 8.89[a] (0.312-127.27) |
| **High-rise apartment** | 3.06[a] (0.37-150.38) | 1.2[a] (0.4-86.44) | 2.15[a] (0.37-150.38) | 18.18[a] (0.37-102) |

*Kruskal-Wallis analysis
Entries with different superscripts are significantly different at $p < 0.05$ by Mann-Whitney analysis of variance for multiple comparisons

**Table 4** Medians (ranges) of Per a 9 in µg/g of dust collected in different seasons from different room types

| Season | Median (range) of Per a 9 in µg/g of dust | | |
|---|---|---|---|
| | Living room/ television watching area | Bedroom/sleeping area | Kitchen/cooking and eating area |
| Summer | 1.64 [a] (0.33-142.00) | 2.08 [a] (0.33-277.00) | 2.65[a] (0.312-229.85) |
| Rainy season | 3.53 [a](0.312-451.29) | 10.59[b] (0.312-524.14) | 8.45[b] (0.312-389.86) |
| Winter | 8.54 [b](0.312-588.67) | 10.37[b] (0.23-313.67) | 8.89[b] (0.33-234.93) |

Entries with different superscripts are statistically different at $p < 0.05$ using Kruskal-Wallis and Mann-Whitney analyses of variance for multiple comparisons

**Table 5** Seasonal levels of Per a 9 (µg/g of dust) in houses with different construction materials

| Season | Median (range) of Per a 9 in µg/g of dust in houses with different construction materials | | |
|---|---|---|---|
| | Wood | Concrete and wood | Concrete |
| Summer | 2.17[a] ( 0.33-175.75) | 1.65[a] (0.312-277.0) | 2.17[a] (0.33-185.75) |
| Rainy season | 7.40[b] (0.51-164.13) | 20.75 [b] (0.312-524.14) | 2.5[a] (0.312-298.50) |
| Winter | 60.74[c] (0.312-255.33) | 13.72[c] (0.312-588.67) | 5.42[a] (0.312-336.36) |

Entries with different superscripts are different at $p < 0.05$ (Mann-Whitney analysis of variance for multiple comparisons).

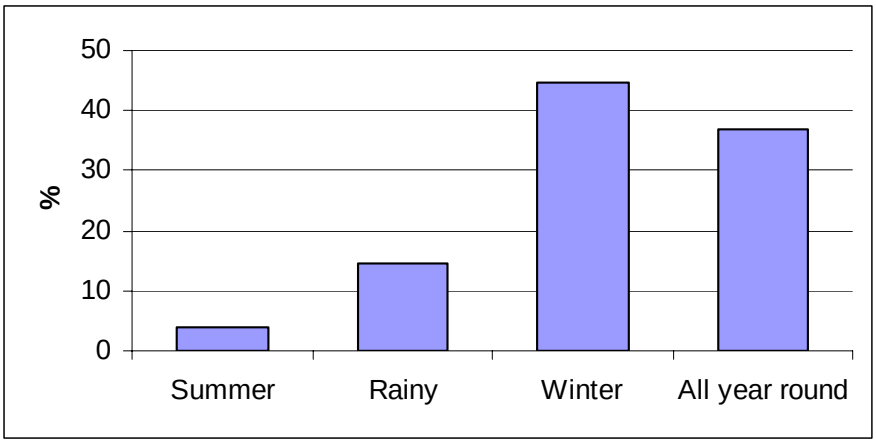


**Fig. 1** The percentage of CR allergic patients reporting their most severe clinical manifestations per season.

in the winter months (Table 5).

Fig. 1 shows which percentage of CR allergic patients reported their most severe clinical manifestations in each season. While ~37% of the patients reported developing the most severe symptoms anytime regardless of the season, ~45 % reported having the most severe symptoms in winter which correlated with the highest levels of CR allergens in most dwellings. Only ~4% of the allergic patients reported having the most severe symptoms in summer when the allergen levels were lowest in most houses and 14% had the most severe allergic symptoms in the rainy season.

## DISCUSSION

Monitoring the levels of cockroach allergen in the environment has medical relevance as a clear dose response relationship between CR allergen ex-

posure and sensitization as well as with asthma and other asthma related morbidity has been reported.[16-18] Exposure to Bla g 1, the major allergen of the German CR (*Blattella germanica*), at 1-2 U/g and 4 U/g of dust has been shown to convert 32% and 40 to 45% of children to positive skin tests to the CR allergen, respectively.[17] Children exposed to CR allergen early in life were associated with recurrent wheezing and asthma later.[19] Moreover, children exposed to > 2 U/g Bla g1 or Bla g2 had a high risk of recurrent asthmatic wheezing. Obviously, avoiding a CR allergen polluted environment is an effective measure for preventing sensitization of naïve individuals and for ameliorating the allergic morbidity of CR sensitized subjects.

Several studies have shown that the highest levels of CR allergen in infested houses were in the kitchens.[7,20] Nevertheless, exposure in bedrooms and living rooms where residents spend more time every day has the best correlation with sensitization.[20,21] Data on the threshold levels of *P. americana* allergens in relation to the allergic sensitization and hospitalized asthma are not available currently. Most studies report on *B. germanica* allergens. Bla g 1 concentrations at 2 U/g and 8 U/g were threshold levels for sensitization and hospitalized asthma, respectively.[17] Atopic children exposed to more than 0.32 mg/g of Bla g 2 dust were sensitized.[19]

Among the *P. americana* allergens, Per a 1 and Per a 9 (arginine kinase) are the major components that bound to IgE in the sera of all CR allergic Thais.[12,13] The levels of Per a 1 in the houses of CR allergic Thai patients were determined in 2004 for the purpose of disease intervention.[7] In the present study, the levels of Per a 9 were quantified in the patients' houses located in the Bangkok metropolitan area in various seasons throughout the year 2007. The relationship of the allergen levels with the types of houses and the construction materials as well as the correlation between the allergen levels and the severity of the allergic symptoms were determined.

The CR allergen was found throughout the year in the patients' houses which conforms to the notion that cockroach allergens are highly mobile and perennial indoor allergens.[20] However, the levels of the Per a 9 showed a somewhat seasonal trend in this study. The highest levels seemed to be prevalent in the winter months while much lower amounts were found in the summer regardless of room types. The levels in the rainy season were comparable to those during winter in the bedrooms/sleeping areas and in the kitchens/cooking and eating areas but were significantly lower in the living rooms/television watching areas. The high amounts of Per a 9 in the winter months in all room types correlated with the allergic disease exacerbation in the majority (~45%) of the CR allergic patients during this time. However, significant proportions of the CR allergic patients, ~37% reported disease severity at any time of the year and 4% during summer when the allergen levels were lowest in the dwellings. This might be due to several other confounding contributors such as the patients' physical activities, respiratory infections, exceptionally high ambient temperature in the summer and other indoor allergens including house dust mites, pets and others.

The allergen levels were significantly higher in the houses made of wood only or of wood and concrete in combination than in the concrete houses. These data indicated that the insects preferred wood-based houses to concrete based-construction.

From the 5 types of houses, *i.e.* isolated houses, mercantile settings, slum huts, town/terrace houses and high rise apartments, it was found that the median of the allergen levels were highest in the slum huts throughout the year as well as during the rainy season and winter. Although there were too few of this type of dwelling in this study, it could be envisaged that the high levels of CR allergens in the slum huts were due to the substandard environmental sanitation and the crowded condition. The CR allergen levels in the mercantile houses at almost any time of the year were the second highest. In fact, the mercantile settings in Thailand are not much different environmentally from the urban slum huts in terms of the crowded situation since individual mercantile sets with multiple stories were attached (shared side-walls) to one another in one building block. The business area of each setting is usually on the ground floor and the merchant family lives in the upper stories. The cockroaches can move freely from one setting to another and can colonize in any place throughout the whole building block. Moreover, certain business types like restaurants and food storages are more prone to CR infestations. The single houses, town/terrace houses and high rise apartments seemed to have lower amounts of Per a 9 al-

lergen most of the year than the slum huts and the mercantile type of dwelling which correlated arbitrarily with the better sanitary conditions and perhaps also the higher economic standard of the owners of the three former compared to those living in the latter two types of houses. Nevertheless, the levels of allergen varied greatly among different single houses from undetectable levels to detectable quantities as high as 588.67 µg/g of dust, implying that the house keeping also regulated the CR allergen levels. In this study, there was a significant portion of houses in which the CR allergens in samples collected from certain rooms at certain times were undetectable. This might be due to the fact that the owners cleaned their houses thoroughly prior to the arrival of the dust collector.

In conclusion, the data of this study show a direct correlation between seasonal levels of the major American CR allergen Per a 9 (arginine kinase) and allergic disease exacerbation of CR allergic patients in Bangkok. CR sensitized subjects are likely to be at a higher risk of severe clinical manifestations, especially asthma, during the winter months and even more so if they live in wood based houses.

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