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# Hypersensitivity Pneumonitis Caused by House Cricket, *Acheta domesticus*

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## Abstract

Hypersensitivity pneumonitis (HP) is characterized by extensive interstitial lung inflammation primarily driven by activated T lymphocytes in response to exogenous antigens. The purpose of this study was to investigate whether chronic exposure to *Acheta domesticus* commonly known as house cricket was the underlying cause of HP in a 63 year old patient. HP was diagnosed based on clinical history, spirometry, high resolution computed tomography (HRCT), bronchoalveolar lavage and trans-bronchial biopsy. Ouchterlony double diffusion assay, direct ELISA and Western blotting were used to detect immuno-reactive precipitins/antigens and anti-cricket antibodies. Mass spectrometry was used to identify the major putative antigens. T cell-mediated response to cricket antigens was assessed by *in vitro* cytokine production assays. HRCT showed extensive bilateral and ground glass opacification throughout the lungs with some sparing at the bases, while bronchoalveolar lavage showed lymphocyte predominant leukocyte infiltration. A lung biopsy showed diffuse chronic interstitial inflammation with poorly defined granulomas consisting lymphocytes and occasional giant cells. There were high titer antibodies against cricket protein extracts and specific antigen-antibody precipitins. Western blotting showed 4 specific immuno-reactive bands. Tryptic peptide digest and mass spectrometry revealed arginine kinase as a potential antigen. Multistep chromatography enriched cricket arginine kinase induced strong *in-vitro* interferon-γ response by PBMC obtained from the patient but not in other cricket-exposed and non-exposed healthy control subjects. This is the first study to report a case of subacute HP in response to prolonged exposure to house cricket antigens.

**Keywords:** Hypersensitivity pneumonitis; House crickets; *Acheta domesticus*; IFN-γ; Mass spectrometry

## Abbreviations

BAL: Bronchoalveolar Lavage; CEF: Cytomegalovirus, Epstein-Barr virus and Influenza virus; DEAE: Dimethylaminoethyl; HP: Hypersensitivity Pneumonitis; PAS: Periodic Scid-Schiff; TMB: Tetramethyl Benzidine

## Introduction

Hypersensitivity pneumonitis (HP), also known as extrinsic alveolitis, is a heterogeneous group of immunologically induced lung diseases resulting from repeated inhalational exposure to a variety of organic and inorganic dusts [1-3]. It is characterised by activated $T_H1$ cells and production of antigen specific antibodies, commonly IgG [2]. HP can be acute, subacute, or chronic depending on the nature of the antigen and the circumstances under which exposure occurs [2,3]. Clinically HP presents with bilateral broncho-alveolitis with dry cough, chest tightness, dyspnea, headaches, and occasional fever chills, myalgia and leukocytosis [4-6]. Deteriorating respiratory function is associated with progressive upper zone radiographic changes including symmetrical homogeneous ground glass opacification and if undiagnosed, it has a potential to progress to end-stage pulmonary fibrosis [7]. Histologically HP is characterized by chronic inflammation of the bronchi and peri-bronchiolar tissue with a predominance of lymphocytes, often with poorly defined non-caseating granulomas, individual giant cells in the interstitium and varying degree of fibrosis [5,8].

Insect dust is a well-known source of allergens in occupational inhalant allergies and asthma [9-14]. Type-I allergic airway diseases characterized by increased IgE production and $T_H2$ mediated responses have been reported in individuals exposed to cockroaches, locusts and house crickets [9-14], and one publication suggesting a HP-like reaction in response to cricket antigens [10]. To our knowledge there are no reports of HP due to chronic house cricket exposure. Importantly, the identities of the specific cricket antigens causing HP or HP-like responses remain unknown. Here we report a case with classical, clinical and histopathological hallmarks of HP in response to chronic exposure to house cricket (*Acheta domesticus*). Moreover, we identified cricket arginine kinase as the potential antigen responsible for the development of this disease. Arginine kinase, also known as ATP-L-arginine omega-N-phosphotransferase, is a 40 kDa phosphagen kinase that is critical for the energy metabolism in insects [15,16]. There are numerous publications describing exposure to arginine kinase from American cockroaches (*Periplaneta americana*) [17], German cockroaches (*Blattella germanica*) [18], Silkworm (*Bombyx mori*) [19] and Indianmeal moth (*Plodia interpunctella*) [20] causing type -I allergic responses [17,20]

but this is the first to report cricket arginine kinase as one of the potential candidates that trigged classical HP.

## Case History

A 63-year-old male ex-smoker presented with a six-week history of marked breathlessness and, associated wheeze and chest tightness. In the last two years, patient had intermittent episodes of cough with pale yellow phlegm lasting for 3-4 weeks but not any other major illnesses. He had previously owned an avian pet shop and owned a cockatoo. For the 6 years prior to presentation herbed thousands of house crickets (*Acheta domesticus*) in boxes as a commercial food source for reptiles. He did not note a specific relationship of his work to the symptoms.

The patient was afebrile but there were bilateral inspiratory crepitation and wheezing in the mid-zones. His oxygen saturation was reduced to 91% at room air and spirometry indicated a restrictive pattern with FEV1 1.63L, 44% predicted; FVC 1.82L, ratio 90%. High resolution chest CT scan demonstrated extensive ground glass opacification throughout both lung fields with some sparing of the bases. Bronchoalveolar fluid revealed a neutrophil-lymphocytosis (50%, 30% respectively). Avian precipitin serology was negative. At the same time, the cockatoo was housed elsewhere and later re-introduced, but this did not precipitate any symptoms. He opted not to take corticosteroids, and instead initiated a reduction in total exposure to crickets in combination with the use of a face mask. After 6 months this led to abatement of the clinical symptoms and improvement of spirometric results (FEV12.92L and FVC 3.54L).

## Experimental Methods

### Histopathology and immunohistochemistry of transbronchial lung biopsy

Six fragments of lung biopsy tissue (1-2 mm in size) were formalin-fixed and paraffin embedded. Four μm sections were then stained with routine hematoxylin and eosin (H&E), reticulin staining and Masson's trichrome. Periodic acidic Schiff (PAS) and Truant auramine-rhodamine staining were used to exclude fungal and mycobacteria infections respectively. Histopathological changes in the lung were assessed independently by two anatomical pathologists. Standard three step immunohistochemical staining was performed to detect T cells, macrophages or cytomegalovirus (CMV) inclusion bodies. In brief, deparaffinised tissue sections were digested with 10 μg/ml of Proteinase K in 0.1 M Tri-HCl containing 50 mM EDTA for 20 min at 37°C and digestion stopped with 0.2% glycine in 0.1 M Tris-HCl followed by two washes in TBS. After blocking non-specific binding using 20% normal goat serum in TBS for 20 min at RT, section were incubated at 4°C overnight with 10 μg/ml of mouse anti-human CD68 mAb (clone: KP1, Dako, Glostrup, Denmark), rabbit anti-human CD3 Ab (clone: UCHT1, Dako), mouse anti-CMV mAb (clone CCH2+DDG9, Dako) or irrelevant control mouse or rabbit Abs in TBS+ 2% BSA. Slides were washed twice in TBS for 5 min and incubated with 5 μg/ml biotinylated goat anti-mouse (Dako) or goat anti-rabbit (Dako) secondary Abs for 1 hour at RT and washed twice in TBS. Alkaline phosphatase (AP)-conjugated avidin-biotin complex (ABC) was added for 45 min at RT, washed twice and finally incubated with ABC-AP substrate 20 min at RT (Vectastain ABC; Vector Laboratories, QLD, Australia). Sections were counterstained with Mayer's haematoxylin (Dako).

### Collection of sera and peripheral blood mononuclear cells

Coagulated and non-coagulated peripheral venous blood was collected from the patient, and an age and gender matched healthy control with no exposure to crickets and two healthy subjects with prolonged exposure to crickets at the same premises. Peripheral blood mononuclear cells (PBMCs) were isolated from acid-citrate-dextrose (ACD) anti-coagulated blood by a standard Ficoll Hypaque density gradient centrifugation (Sigma, NSW, Australia). Sera were extracted from clotted blood by 12,000 ×g for 5 min centrifugation. The study was approved by the institutional human ethics committee and informed consent was obtained from all subjects.

### Extraction of crude house cricket proteins

The abdominal/thoracic walls, the guts, wings/legs, and hearts of each insect were carefully dissected. One gram of tissue from each component or from the whole insect were frozen at -80°C and crushed with a mortar and pestle followed by homogenization in cold RIPA buffer (Sigma) using an ultrasonic cell disrupter (3 times for 30 sec at 50% power cycle, Kontes, NJ, USA). Soluble homogenized proteins were extracted by high speed centrifugation at 12,000 ×g for 30 min, and concentrations in the supernatant determined by BCA protein assay (Thermo Scientific, VIC, Australia). Approximately 10 mg of protein was obtained from 5 whole insects and 15-20 insects were required to extract 3-7 mg of protein from the abdominal/thoracic walls, the guts, wings/legs, or hearts.

### Immunodiffusion assay for cricket antigen precipitins

An immunodiffusion assay using protein extracted from whole insects and patient serum was performed as described [21]. Briefly, 1.5% agar was prepared in 50 ml of 0.85% NaCl and 1% PBS (pH7.4), and 5 ml aliquots dispensed onto 35 mm Petri dishes (Falcon Plastics) and allowed to solidify at 4°C. Three 7 mm equidistant wells were punched out in a row, the center wells filled with 50 μl of serum from the patient or the healthy control subject (1 in 10 dilution in saline). Remaining cells on the left were filled with 0.6 mg of whole cricket protein extract in 50 μl of saline and wells on the right were filled with 50 μl of saline as no protein controls. The plates were then incubated for 24 hours at RT with wells refilled 4 times with 50 μl of sera, cricket protein or saline. Lines of precipitation indicated a positive result.

### Detection of antibodies against cricket proteins in sera by direct ELISA

Flat-bottom 96 well polystyrene plates (Nunc Maxisorb CA, USA) were pre-coated with 100 μl of 0.05 M carbonate-bicabonate, pH 9.6 for 30 min at RT. This was followed by coating duplicate wells with 100 μl (10 μg) of crude cricket protein extracts from the abdominal/thoracic walls, the guts, wings/legs, or hearts and an overnight incubation at 4°C. Unbound proteins were aspirated and wells blocked with 200 μl of 5% skim milk in PBST (PBS buffer with 0.05% Tween 20, pH 7.4) for 30 min at RT, and washed 3 times with 200 μl of PBST (PBS buffer with 0.05% Tween 20, pH7.4). Serial dilutions of patient or control sera in 5% skim milk in PBST were then added to duplicate wells and plates incubated at 4°C overnight. The next day, plates were washed 3 times with 200 μl of PBST and incubated with 0.2 μg/ml of HRP conjugated mouse anti-human antibody in TBS (Jackson

Immuno Research, PA, USA) in the dark for 2 hours at RT. After 3 more washes with PBST, TMB chromogen substrate (Nalgene, Panbio, QLD, Australia) was added. The reaction was stopped by adding 50 µl of 1M $H_2SO_4$ after 60 min incubation at RT in the dark and optical density measured at 450 nm with the SpectraMax® M2 Microplate Reader (Molecular Devices, CA, USA). Initially, titration of anti-cricket antibodies against the different components of the insect was determined using 50 µl of serially diluted patient or control sera (ranged from $1:10^1$ to $1:10^8$ dilutions in 5% skim milk in PBST) to duplicate wells. In subsequent experiments, $1:10^4$ diluted sera and proteins extracted from the wings/legs and hearts were used.

## Western blotting and silver staining of cricket-derived proteins

Protein extracts (20 µg) from the whole cricket or the different parts of the insect were heated at 100°C for 5 min with 10 mM DTT (Sigma, NSW, Australia) and separated using 10% SDS PAGE gels. To perform western blotting, the proteins were transferred onto polyvinyldifluoride membranes (PVDF, 0.2 µm pore size, Millipore, VIC, Australia), washed in TBS for 20 min, then blocked in 5% skim milk at 4°C overnight. Following 3 washes with TBST (TBS + 0.05% Tween 20), membranes were incubated for 2 hours at RT in patient sera or sera from a healthy control subject diluted 1:30 in TBS. Unbound sera were washed 3 times with TBST and membranes incubated with 0.1 µg/ml horseradish peroxidase (HRP) conjugated mouse anti-human IgG (H+L) (Jackson Immuno Research) for 90 min at RT. This was followed by a further 3 washes with TBST. Detection and image capture used Western Lighting chemiluminescent reagent (PerkinElmer, VIC, Australia) and ImageQuant™ LAS 4000 (GE Healthcare Life Sciences, NSW, Australia) respectively.

A second SDS PAGE gel was run in parallel to each gel used for a western blotting and silver stained as described [22]. Briefly, gels were fixed with 50% methanol/5% acetic acid for 10 min followed by 50% methanol only for 10 min. Gels were then washed twice in distilled water for 10 min, incubated in 0.05% sodium thiosulfate solution for 1 min, rinsed in distilled water and incubated in 0.2% silver nitrate for 1 hour. This was followed by a quick rinse in distilled water, detection of reactive bands using 2% sodium carbonate+0.04% formalin. Silver stained bands that matched the size of the immuno-reactive bands on the corresponding Western blotted membrane were excised and sent for peptide mass spectrometric sequencing.

## Identification of immuno-reactive proteins by mass spectrometry

In brief, 4 specific immuno-reactive bands at approximately 160, 130, 75 and 65 kDa were detected by Western blotting of crude protein lysates from the whole insect or body parts using the patient's but not the control's serum. Silver stained bands that correspond to these sizes were excised, tryptically in-gel digested and separated by nano-LC using an Ultimate 3000 HPLC and auto sampler system (Dionex, Amsterdam, Netherlands). Samples were then concentrated and desalted onto a micro C18 pre-column with $H_2O$:$CH_3CN$ (98:2, 0.05 % TFA) at 15 µl/min. After a 4 min wash the pre-column was switched into line with a fritless nano-column (75µ x ~10 cm) containing C18 media [22,23]. Peptides were eluted using a linear gradient of $H_2O$:$CH_3CN$ (98:2, 0.1 % formic acid) to $H_2O$:$CH_3CN$ (64:36, 0.1 % formic acid) at 250 nl/min over 30 min. Positive ions were generated by electrospray and the Orbitrap Velos mass spectrometer operated in data dependent acquisition mode (DDA, Thermo Electron, Bremen, Germany). Peak lists were generated using Mascot Daemon/extract_msn (Matrix Science, Thermo, London, England) using the default parameters, and submitted to the database search program (Mascot version 2.2, Matrix Science). Search parameters were: precursor tolerance 4 ppm and product ion tolerances ± 0.4 Da; oxidation (M) and carbamidomethyl (C) specified as variable modifications, the enzyme specificity was trypsin, 1 missed cleavage was possible and the S-Prot protein database (August 2012) with *Homo sapiens* as the taxonomy.

## Enrichment of cricket arginine kinase from cricket wings and legs

The proteins extracted from wings and legs were found to be the most immuno-reactive to the patient sera. Arginine kinase, a known insect allergen, was identified as a strong candidate by mass spectrometry with high Mascot scores and multiple peptide matches (Mascot score 200-384 with 5-11 peptide matches). An established protocol was used to enrich for arginine kinase from the wings and legs [24,25]. In brief, a total of 8 grams of cricket wings and legs were dissected from 200 fresh frozen house crickets, minced in 40 ml of buffer A (0.1 M Tris/HCl, 10 µM 2-mercaptoethanol, 1 mM EDTA, 50 µM $NaN_3$, pH 8.0) and sonicated as described above. Protein lysate was then concentrated by Amicon YC cellulose ultra-filtration membrane with a 10,000 Dalton cutoff (Millipore, VIC, Australia). Concentrated protein was first loaded onto a pre-packed 1.6 cm × 60 cm Superdex 200 preparation grade gel column (GE Healthcare Life Sciences, NSW, Australia) equilibrated with buffer B (10 mM Tris/HCl, 10 µM 2-mercaptoethanol and 0.1 mM EDTA, pH 8.0) followed by elution of 27x 1 ml fractions containing proteins ranging from 3 to 70 kDa. These were immunoblotted using patient sera and fractions containing immunoreactive proteins were combined and re-loaded onto 1.5 ml DEAE Sepharose gel (GE Healthcare Life Sciences, NSW, Australia) in a 0.7 cm×15 cm Grass Econo-Column® (Bio-Rad Laboratories, CA, USA). Unbound proteins were then washed with 15 ml (10 bed volumes) of buffer B followed by elution of bound proteins using buffer B containing 0.5 M NaCl in 4x 1 ml fractions. Each fraction was buffer exchanged into PBS by dialysis and protein concentrations determined by ND-1000 spectrophotometer (NanoDrop, NC, USA). The presence of enriched immunoreactive proteins was confirmed by western blotting using patient sera and the identity of immunoreactive arginine kinase was confirmed by peptide sequencing using mass spectrometry.

## Multiplex cytokine assay and IFN-BMCs

PBMC from the patient and control subjects were cultured in RPMI 1640 (supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin; Sigma, NSW, Australia) in a 96 well flat bottom plates at $2\times10^6$/ml in duplicates (200 µl/well) with or without 2.5 µg/ml of crude whole cricket protein extracts or cricket proteins from the wings and legs that were enriched for arginine kinase. After incubation in 5% $CO_2$, at 37°C for 72 hours, culture supernatants were collected. Cytomegalovirus, Epstein-Barr virus and Influenza virus (CEF) peptides (National Institute of Health, MD, USA), 5 µg/ml, and $4\times10^5$ of anti-CD3/CD28 Dynabeads (Invitrogen, CA, USA) were used as positive controls. A Bio-Plex assay was used for initial screening of TNF-α, IL-2, IL-4, IL-5, IL-10, IL-12 and IFN-γ levels in culture supernatants (Bio-Rad Laboratories, CA, USA). Production of IFN-γ

was further confirmed by a commercial sandwich ELISA kit (R&D System, MN, USA).

## Results

### Trans-bronchial lung biopsy showed histopathology and immunohistochemistry consistent with HP

Sectioning at multiple levels showed bronchial mucosa with mild acute-on-chronic inflammation. The adjacent parenchyma showed prominent expansion of the alveolar interstitium by a prominent lymphohistiocytic infiltrate. One of the fragments showed lung with relatively preserved alveolar architecture (Figure 1A). Mild smooth muscle hypertrophy, mild anthracosis and multinucleated giant cells containing cholesterol crystals (Figure 1B) were present. A poorly formed granuloma was seen in the interstitium (Figure 1C). Pneumocytes lining the alveoli showed reactive/hyperplastic changes (Figure 1D). Immature fibrous tissue was focally present in the interstitium as well as within the lung alveoli as shown by H&E staining (Figure 1D) and confirmed by Masson's trichrome and reticulin staining (not shown). Immunohistochemical staining showed a large number of $CD3^+$ T cells (Figure 1E) and $CD68^+$ macrophages (Figure 1F). No fungi, mycobacteria or CMV inclusions were found (not shown).

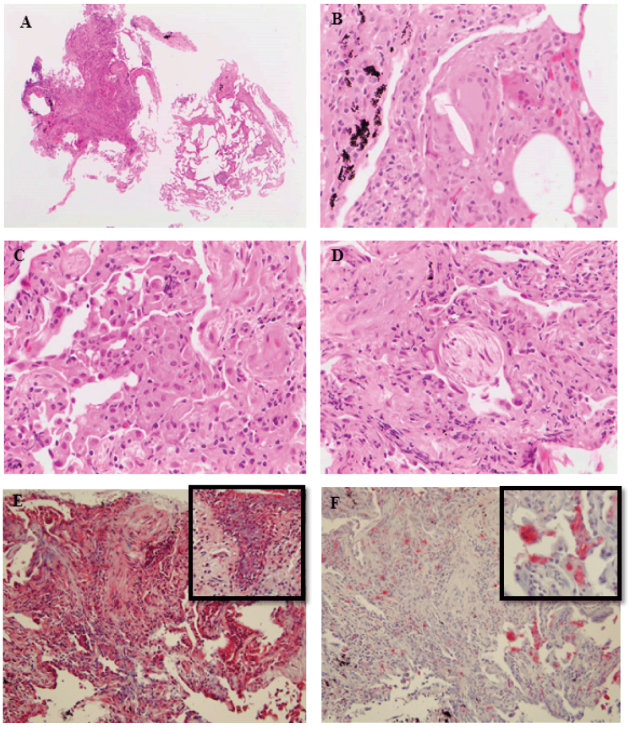


**Figure 1: Histology and histochemistry of lung biopsy: A.** H&E of one of the six biopsies showing relatively well preserved alveoli. **B** and **C** peripheral lung showing a giant cell, poorly formed granulomas and lymphohistiocytic infiltrate. **D.** Immature fibrous tissue in the interstitium and alveoli. Immunohistochemistry using anti-CD3, **(E)** or anti-CD68, **(F)** monoclonal antibodies showing abundant presence of T cells and macrophages respectively.

### Patient sera contained high titer antibody against cricket antigens

Immunodiffusion using the patient sera but not sera from a healthy subject showed strong immunoprecipitin bands against whole cricket extracts (Figure 2A), indicating the presence of cricket antigen-specific antibodies in patient sera. No precipitins were observed between patient sera and avian antigens confirming specificity (not shown).

Direct ELISA using serially diluted patient and a healthy subject sera and plate immobilized cricket proteins demonstrated a strong reaction against protein extracted from the wings/legs and thoracic walls in patient's sera at 1:10,000 dilution but not in control sera (Figure 2B and 2C). By contrast, there was little or no immunoreaction against the protein extracted from the internal organs of the insects (gut or heart) against sera from the patient or control subject (Figure 2B and 2C). Interestingly, antibodies against the protein extracted from the wings/legs of the insect in patient's sera remained persistently high over a period of 3 years, despite a reduction in occupational exposure (Figure 2D).

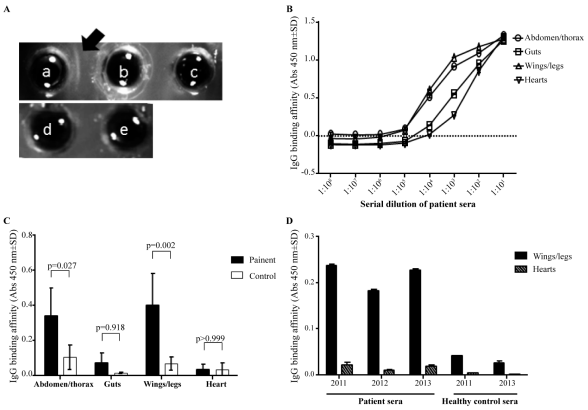


**Figure 2: Detection of anti-cricket antibodies and precipitins between patient sera and cricket antigen: A.** Immunodiffusion betweenwhole cricket extracts (a) and the patient sera (b) compared with a negative control (c) and also between whole cricket extracts (d) and normal control sera (e). Arrow indicates antigen-antibody lines of precipitation. Arrow indicates antigen-antibody lines of precipitation. **B.** Titration of anti-cricket antibodies showing the highest titer of antibody against the wings/legs and abdominal/thoracic wall extracts. **C.** Comparative binding to cricket antigens demonstrating significantly higher affinity binding of patient sera than a control subject to wings/legs and abdominal/thoracic wall proteins (n=3). **D.** Persistent high levels of antibodies against wings/legs of cricket in patient sera overtime. Two-way ANOVA with Sidak's multiple comparisons tests was used for statistical analysis.

### Western blot demonstrated four specific immuno-reactive cricket antigens

Immunoblotting of crude cricket protein extracts (20 µg each) with patient serum but not serum from a healthy control showed four immuno-reactive bands at approximately 160, 130, 75 and 65 kDa

(Figure 3A). These immuno-reactive bands were most prominent in the lane loaded with protein extracted from the wings and legs (Figure 3A, lane 8). This is consistent with the high titre of antibody observed against these parts of the insect (Figure 2). Semi-purification of the proteins extracted from the wings and legs using Superdex 200 gel chromatography markedly enhanced the intensity of all four prominent immuno-reactive bands in multiple elusion fractions (Figure 3B). This was despite the use of 20 fold (1 µg) less protein as compared to the crude whole protein extract, suggesting significant enrichment of the specific antigens. Further purification of the gel chromatography enriched protein using DEAE column selectively augmented the immuno-reactivity of only the160 kDa and the 65 kDa proteins (Figure 3C).

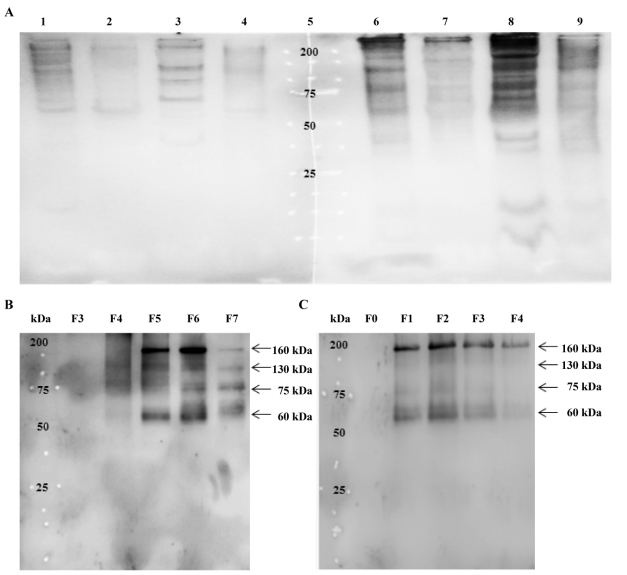


**Figure 3: Detection of immuno-reactive cricket proteins to patient sera:** A representative western blot of crude protein extracts from different body parts of the insect (20 µg each) blotted with patient sera (lanes 6-9) showed more intense immuno-reactive bands as compared to lanes blotted with control sera (1-4) **(A)**; lanes 1and 6, abdomen/thorax; lanes 2 and 7, guts; lanes 3 and 8, wings/legs; lanes 4 and 9, hearts (n=3). Protein extracted the most immuno-reactive component of the insect (wings/legs, see A) was semi-purified by a single step chromatography using Superdex 200 **(B)** or two-step chromatography using Superdex 200 followed by DEAE Sepharose **(C)** 1 µg fractions blotted with patient sera. The four prominent bands shown in Figure A are enriched upon purification despite the use of 20 fold less protein. Figure B and C are representative of 2 independent experiments.

## Peptide sequencing of immunoreactive cricket antigens by mass spectrometry identified arginine kinase as the most abundant protein

To determine the identities of the cricket antigens to which the patient sera reacted, we first performed in-gel tryptic digest and peptide sequencing mass sequencing of the bands from crude protein extracts of the whole insect. All four immuno-reactive bands revealed the presence of arginine kinase with high Mascot scores and multiple peptide matches (Table 1), suggesting a monomeric (65-75 kDa), dimeric (130 kDa) and tetrameric (160 kDa) forms of this protein. As expected, two-step enrichment using gel and DEAE chromatography substantially increased the Mascot scores and the number of peptides that matched arginine kinase by up to 3 fold (Table 2). By contrast, a number of proteins that were detected in the initial sequencing of the crude cricket extract including myosin, paramyosin, tublin, and various forms of ATP synthases and translocases were either totally depleted or significantly decreased following chromatography (Table 3). Interestingly, peptide sequencing of the semi-purified proteins identified proteins that were not evident in the initial sequencing of the crude protein extracts, some of which are known insect allergens [17,20,26-28] (Supplementary Table 1). These include α actinin, heat shock proteins 70 and 90, tropomyosin and myosin.

| Mass in kDa | Protein | Mascot Score | Peptide Matches |
|---|---|---|---|
| 160 | Myosin heavy chain | 3852 | 98 |
| | Sar/endoplasmic reticulum calcium ATPase | 972 | 25 |
| | Actin | 748 | 25 |
| | Paramyosin | 316 | 8 |
| | Tubulin alpha | 287 | 7 |
| | ADP/ATP translocase | 270 | 6 |
| | ATP synthase beta subunit | 249 | 5 |
| | Adenine nucleotide translocase | 221 | 5 |
| | **Arginine kinase** | **200** | **5** |
| 130 | Myosin heavy chain | 2853 | 69 |
| | Sar/endoplasmic reticulum calcium ATPase | 1200 | 37 |
| | Paramyosin | 779 | 20 |
| | Actin | 544 | 16 |
| | **Arginine kinase** | **193** | **5** |
| | ADP/ATP translocase | 169 | 5 |
| | Mitochondrial ATP synthase alpha-subunit | 162 | 3 |
| 75 | Myosin heavy chain | 1285 | 24 |
| | Actin | 565 | 16 |
| | Sar/endoplasmic reticulum calcium ATPase | 565 | 11 |
| | Paramyosin | 186 | 4 |
| | **Arginine kinase** | **184** | **6** |
| | Sodium/potassium-transporting ATPase | 160 | 4 |
| | Tropomyosin | 154 | 3 |
| 65 | Myosin heavy chain | 1200 | 27 |
| | ATP synthase subunit alpha | 938 | 24 |

| | | | |
|---|---|---|---|
| | Tubulin beta | 834 | 32 |
| | Actin | 830 | 32 |
| | Tubulin alpha | 493 | 12 |
| | Troponin | 476 | 18 |
| | **Arginine kinase** | **383** | **11** |
| | ATP synthase subunit beta | 316 | 7 |

**Table 1:** Mascot search results of mass spectrometric peptide sequencing of the immuno-reactive bands detected upon blotting of crude whole cricket protein extracts with patient's sera.

| Mass in kDa | Arginine kinase | Before chromatography | After chromatography |
|---|---|---|---|
| 160 | Mascot Score | 200 | 404 |
| | Peptide Matches | 5 | 14 |
| 130 | Mascot Score | 193 | 414 |
| | Peptide Matches | 5 | 13 |
| 75 | Mascot Score | 184 | 393 |
| | Peptide Matches | 6 | 11 |
| 65 | Mascot Score | 383 | 356 |
| | Peptide Matches | 11 | 9 |

**Table 2:** Marked increase in Mascot scores and the number of peptide matches to arginine kinase in gel-chromatography-enriched cricket wings/legs proteins.

| Mass in kDa | Protein | Mascot Score | | Peptide Matches | |
|---|---|---|---|---|---|
| | | Before chromatography | After chromatography | Before chromatography | After chromatography |
| 160 | Myosin heavy chain | 3852 | 605 | 98 | 17 |
| | Sar/endoplasmic reticulum calcium ATPase | 972 | 0 | 25 | 0 |
| | Actin | 748 | 0 | 25 | 0 |
| | Paramyosin | 316 | 0 | 8 | 0 |
| | Tubulin alpha | 287 | 267 | 7 | 7 |
| | ADP/ATP translocase | 270 | 0 | 6 | 0 |
| | ATP synthase beta subunit | 249 | 0 | 5 | 0 |
| | Adenine nucleotide translocase | 221 | 0 | 5 | 0 |
| 130 | Myosin heavy chain | 2853 | 1337 | 69 | 32 |
| | Sar/endoplasmic reticulum calcium ATPase | 1200 | 0 | 37 | 0 |
| | Paramyosin | 779 | 0 | 20 | 0 |
| | Actin | 544 | 0 | 16 | 0 |
| | ADP/ATP translocase | 169 | 0 | 5 | 0 |
| | Mitochondrial ATP synthase alpha-subunit* | 162 | 216 | 3 | 5 |
| 75 | Myosin heavy chain | 1285 | 965 | 24 | 18 |
| | Actin | 565 | 0 | 16 | 0 |
| | Sar/endoplasmic reticulum calciumATPase | 565 | 0 | 11 | 0 |
| | Paramyosin | 186 | 0 | 4 | 0 |
| | Sodium/potassium-transporting ATPase alpha chain | 160 | 0 | 4 | 0 |
| | Tropomyosin** | 154 | 151 | 3 | 3 |
| 65 | Myosin heavy chain | 1200 | 971 | 27 | 14 |
| | ATP synthase subunit alpha | 938 | 0 | 24 | 0 |
| | Tubulin beta | 834 | 181 | 32 | 6 |
| | Actin | 830 | 0 | 32 | 0 |
| | Tubulin alpha | 493 | 0 | 12 | 0 |
| | Troponin | 476 | 0 | 18 | 0 |
| | ATP synthase subunit beta | 316 | 0 | 7 | 0 |
| *enriched after chromatography; **no change after chromatography | | | | | |

**Table 3:** Total depletion or substantial decrease in Mascot scores and the number of peptide matches following gel-chromatography to proteins initially detected in immuno-reactive crude protein extracts.

### *In vitro* stimulation of PBMCs with chromatography-enriched cricket proteins induced selective cytokines in the patient but not a healthy control subject

Screening of culture supernatants from antigen stimulated patient PBMCs for multiple cytokines using multiplex bead assay showed selective up-regulation of IFN-γ and TNF-α (Figure 4) but not IL-4, IL-5 or IL-12 (not shown), indicating a $T_H1$ biased response. Stimulation of patient's PBMC with Superdex and DEAE chromatography-purified insect extract that was enriched for arginine kinase substantially increased IFN-γ (2-3 fold) and TNF-α (3 fold) production as compared to cells stimulated with the same concentrations of crude insect protein lysate (Figure 4A-4D). The increased production of IFN-γ by PBMCs from the patient after *in vitro* activation with cricket antigens was repeatedly confirmed by

ELISA (Figure 5). Consistent with the multiplex cytokine assay, treatment of patient's PBMC with arginine kinase-enriched cricket extracts using DEAE induced 4 times more IFN-γ (123.8 ± 4.38 pg/ml) when compared to cells treated with the same amounts of single step Superdex GEL semi-purified cricket lysates (33.1 ± 2.16 pg/ml, Figure 5). There was no or little IFN-γ production by patient's PBMC in response to buffer controls, indicating specificity (Figure 5). Interestingly, 1 year after decreased exposure to the insects, the response to arginine kinase-enriched cricket protein by the patient's PBMC remained high with 79.3 ± 3.14 pg/ml of IFN-γ production as compared to 9.63 ± 5.70 pg/ml in buffer control (Figure 5). No IFN-γ was detected in supernatants of cricket antigen-stimulated PBMC from the healthy control subject (below detection level) while little (<10 pg/ml) was detected in two other individuals who were occupationally exposed to crickets for several years but who did not have clinical symptoms (data not shown).

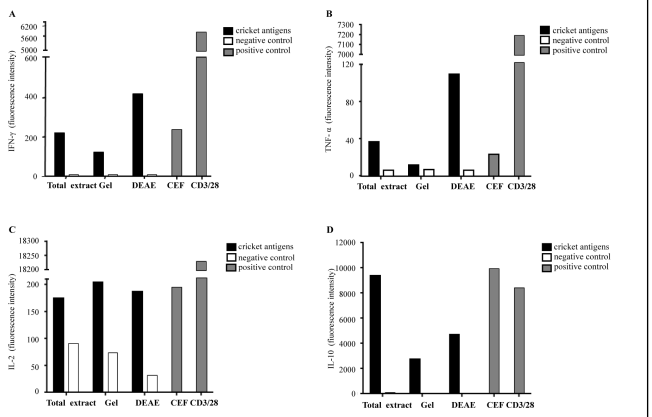


**Figure 4: Cytokine multiplex assay:** *Ex-vivo* stimulation of PBMCs with extracts from wings and legs, purified extractsfromgel chromatography, DEAE chromatography, positive controls (CEF and CD3/CD28) or individual negative controls (Total extract: lysis buffer, Gel: buffer B or DEAE: PBS) was screened by multiplex cytokine bead assay in duplicatesof a single experiment. Different levels of fluorescence intensity (FI) of IFN-γ **(A)**, TNF-α **(B)**, IL-2 **(C)** and IL-10 **(D)** are shown.

## Discussion

Hypersensitivity pneumonitis is a complex disease that can be challenging to recognize. Without a meticulous clinical and histological assessment and high level of suspicion, it can be easily overlooked and/or misdiagnosed as another type of inflammatory lung disease. Hence, a diagnosis of HP requires multiple approaches including clinical, radiological and pathological findings. This approach was particularly relevant to our patient as the offending antigen was not known to be associated with this disease [29]. Sensitization to cricket antigens has previously been reported in relation to asthma and IgE-mediated Type I hypersensitivity reactions [10-14]. However, the typical history of long occupational exposure to house cricket and the insidious onset of breathlessness, fatigue and cough over several weeks are consistent with Type III and/or Type IV subacute HP due to repeated exposure to low-level antigens [30]. Thus the initial clinical impression was further supported by radiological features characterized by ground-glass appearance on the high-resolution CT scan [31], decreased lung function with a restrictive pattern [32] and lymphocytosis in BAL [33]. Importantly, the histopathological findings on the lung biopsies showing bronchiolo-alveolar fibrosis and poorly formed granulomas with no fungi, mycobacteria or CMV inclusions are highly consistent with subacute HP [34]. Although the symptoms, histology and radiological findings can overlap with other interstitial pneumonias [33], the presence of a positive precipitin reaction and high levels of circulating antibodies against the house cricket proteins together with the clinical improvement upon reduced exposure observed in this patient strongly corroborated the diagnosis of HP.

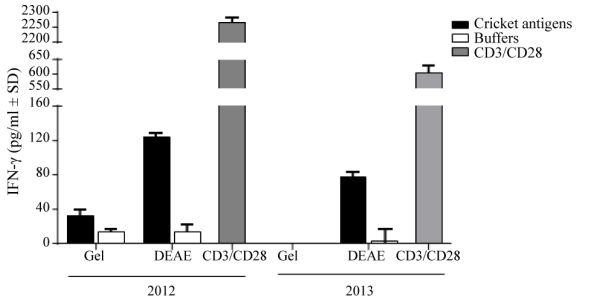


**Figure 5: IFN-γ ELISA:** High level of IFN-γ production in response to arginine kinase-enriched cricket extracts was validated by ELISA using PBMC of the patient collected at two different time points (2012 and 2013) using two independent antigen purifications. Each experiment was performed twice in duplicate wells and values represent Mean ± SD.

Subacute HP is known to respond well to treatment with corticosteroids and removal of the offending antigen but unrecognized disease may progress into chronic disease with life threatening consequences [30]. This patient preferred to use respiratory precautions and evaluate the response rather than to take corticosteroids. Thus a physician must be acutely aware of its existence and undertake detailed occupational and home environment history. Strict avoidance of the provocative antigen is required for optimal long-term clinical outcome [30] but in some cases the offending antigen cannot be identified. In this study we identified a number of candidate cricket antigens using a combination of immunochemical and proteomic approaches, primarily from the wings and legs (Table 1). One of the most frequently and abundantly identified immuno-reactive proteins was arginine kinase (Table 1). Insect arginine kinase, a known allergen in other insects, e.g. cockroaches and locusts, is an approximately 40 kDa [17,35] but its actual mass in house cricket is unknown. We identified multiple peptides matching arginine kinase at 65-70 kDa, 130 kDa and 160 kDa, suggesting a slightly larger monomeric protein in house cricket that was readily dimerized and tetramerized to 130 kDa and 160 kDa mass. Interestingly, arginine kinases from various other insects are primarily associated with Type I hypersensitivity [17,19,36], while our findings including the robust IFN-γ production in response to semi-purified arginine kinase *in vitro* (Figure 4) are consistent with Type III and/or Type IV immunological reaction [37,38]. It is possible that unlike arginine kinases from other insects, the house cricket antigen may have skewed response towards

HP due to its yet undetermined structure and mass. It is also possible that the dose, intensity and frequency of antigenic exposure [39] and genetic predisposition of an individual [40,41] may determine clinical outcome. There are some animal studies that suggest high dose intense allergen exposure may result in Type III and/or IV reactions with HP-like features [39], while chronic low-dose exposure is more likely to result in Type I reaction with asthma-like phenotype [39]. It is noteworthy that the peptide mass sequencing also identified a number of other known antigenic proteins that may have contributed to the disease process (Tables 1 and 3). These include myosin [42], paramyosin [43], actinins [44], tropomyosin [45,46] and various ATPases [47] (Tables 1,3). These insect proteins individually are likely to provoke IgE-mediated allergic reactions or Type I hypersensitivity reactions [42-49] but it is not known to whether these antigens in combination with arginine kinase altered the clinical response in this patient. Lack of recombinant protein and stringent purification of the individual native protein has precluded detailed analysis of their relative contributions. However, enrichment of arginine kinase was associated with increased immuno-reactivity to patient sera (Figure 3) and more robust *in vitro* IFN-γ response (Figure 5) indicating its potential major role in this patient's condition. Interestingly, decreased exposure to the insect has resulted in marked amelioration of the patient's clinical conditions and in marked decrease in IFN-γ production (from 123.8 to 79.3 pg/ml) in response to *in vitro* challenge of his PBMC with semi-purified antigens.In the future N-terminal protein sequencing and/or gene cloning, production of recombinant proteins and/or improved purification of these native proteins may shed light to their relative antigenicity *in vitro* and *in vivo*. Moreover, the specificity of house antigens including arginine kinase in provoking a clinically distinct HP in this patient would be strengthened by the inclusion of other patients with HP that had no chronic exposure to this insect as relevant controls. Taken together, this is the first documented report of HP in response to prolonged exposure to large numbers of house cricket in a confined space shedding antigens including arginine kinase through preening of their wings and legs.

## Disclosure

The authors declare no conflicts of interest.

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