

RESEARCH LETTER

The cockroach allergen-like protein is involved in primary respiratory and food allergy to yellow mealworm (*Tenebrio molitor*)

Stefano Nebbia¹ | Cristina Lamberti¹  | Veronica Giorgis² | Maria Gabriella Giuffrida¹ | Marcello Manfredi³ | Emilio Marengo³ | Enrica Pessione⁴ | Achille Schiavone⁵ | Monica Boita² | Luisa Brussino² | Laura Cavallarin¹ | Giovanni Rolla²

¹Institute of Science of Food Production, National Research Council, Grugliasco, Italy

²S.S.d.D.U. Allergologia e Immunologia Clinica A.O. Ordine Mauriziano Umberto I, University of Turin, Turin, Italy

³Center for Translational Research on Autoimmune and Allergic Disease-CAAD, University of Piemonte Orientale, Novara, Italy

⁴Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy

⁵Department of Veterinary Sciences, University of Turin, Turin, Italy

Correspondence

Cristina Lamberti, Institute of Science of Food Production (ISPA), National Research Council (CNR), Largo Paolo Braccini 2 (10095), Grugliasco, Italy.
Email: cristina.lamberti@ispa.cnr.it

To the Editor,

Insects have been proposed as a high quality, efficient and sustainable alternative protein source for humans and animals, and a vast selection of edible products is currently available in many European countries. However, respiratory allergy among professional and domestic breeders and food allergy among consumers are known.^{1,2}

We here report the case of 2 patients (Pt#1 and Pt#2, both male, 24 and 27 years old, respectively) employed in the production of insect flour made of yellow mealworm larvae (*Tenebrio molitor* larvae, TML) and black soldier fly larvae (*Hermetia illucens* larvae, HIL). The patients did not show the previous history of food or respiratory allergies. In the same factory, a total of ten workers handled TML, eight out of ten without developing any symptom. The first symptoms of the 2 patients (rhinoconjunctivitis, itching and contact erythema) appeared after some weeks of repetitive exposure to TML. They did not report any symptoms when exposed to HIL. The symptoms were particularly severe during the sifting of TML from their faeces (TMF), and when the patients entered the rearing room after the sifting operation, they disappeared after 24 hours. The sensitization therefore presumably occurred through a percutaneous route and/or by inhalation. After developing symptoms, Pt#1 continued to work using adequate protection devices, while Pt#2 left the job. The 2 patients were used to eat such edible insects as the greater wax moth, crickets and HI. They experienced an oral allergy syndrome (OAS) characterized by oral pruritus and tightness in the throat, as soon as they start eating for the first time a TML

hamburger. Symptoms recovered spontaneously in about 40 minutes. Following this episode, they continued to tolerate other edible insects, but never tried to taste TML again and refused to undergo provocation test.

In order to characterize the patients' allergic profile, we performed: skin prick tests (SPTs) with a standard battery of inhalant and food allergens, specific IgE for TM, Der p10, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and Prawn (ImmunoCap Thermo Fisher Scientific Inc) and basophil activation test (BAT) with TML and TMF protein extracts. Immunoblot analyses of both protein extracts and LC-MS/MS analysis followed by protein database screening were performed in order to identify the allergens involved in the TM sensitization (see Appendix S1).

The SPTs with inhalant and food allergens were negative in both patients, except for Grass in Pt#1 and Alternaria in Pt#2. Specific TM IgEs were present in both patients (21.00 and 10.63 kU_A/L, respectively), while no specific IgEs were found for house dust mites, Prawn and Der p10. BAT were positive in both subjects for TMF and TML (Figure 1).

Immunoblotting showed a 15 kDa reactive band in both the TML and TMF extracts, when incubated with both patients' sera (Figure 2, panel A). Three additional aspecific bands were also detected in the healthy control patients' serum pool, but only in the TML extract (Figure 2). The cockroach allergen-like protein (CAL) was identified in the TMF sample, while, in addition to CAL, Troponin C (TnC, from *Tribolium castaneum*) and the 86 kDa early-staged encapsulation protein (ESE) were identified in the TML extract (Figure 2, panel B).

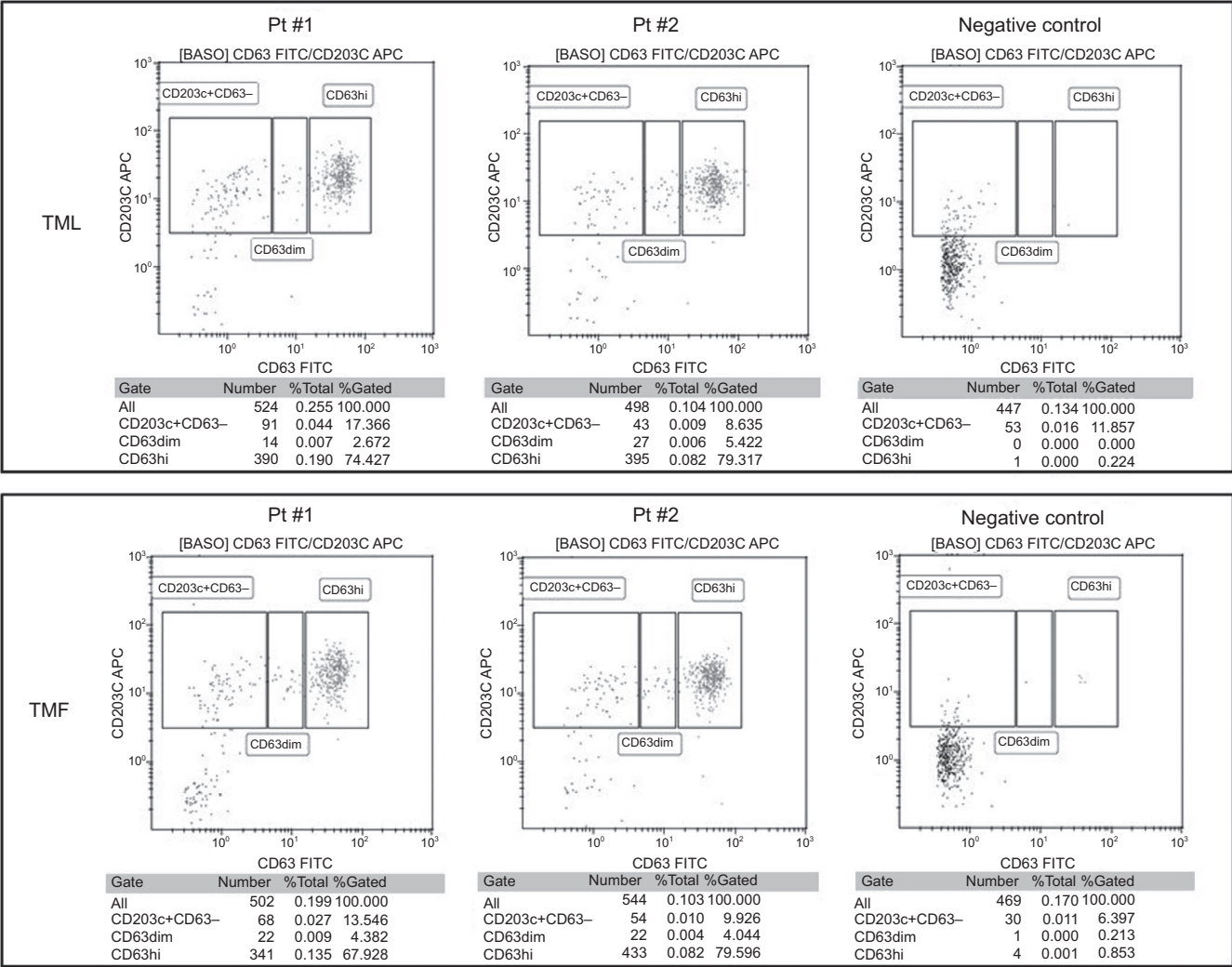


FIGURE 1 BAT with TML and TMF for Pt#1 and Pt#2 compared with a negative control

Occupational allergies following inhalation and/or contact exposure to TM have been reported in several case reports.^{1,2} Broekman et al.² described the case of 4 breeders who developed respiratory allergy to TML, two of whom had a mild allergic reaction after eating TML. IgE immunoblot showed that all the subjects recognized several panallergens (eg, tropomyosin, arginine kinase) but also a larval cuticle protein, which seemed to be the most probable dominant allergen, as the four subjects had a primary sensitization to TML and tolerated crustaceans.

In our 2 patients, IgE binding was shown towards TnC and ESE in TML, and towards CAL in both TML and TMF. TnC is an Arthropoda panallergen and has been characterized as a minor allergen in shrimps,³ whereas it is an important cockroach allergen (Bla g6, Per a6).⁴ Bla g6 from *Blattella germanica* showed a 78.84% identity with TnC from TM (<https://www.uniprot.org/blast>).

Early-staged encapsulation protein is a defence response protein against invading parasites and pathogens, and it requires subsequent cutting steps to be activated.⁵ We found a degraded fragment of this molecule with a mass of about 15 KDa, corresponding to the

C-terminal Haemocyanin domain. Haemocyanin has already been reported to be a minor allergen in crustacean allergies.⁶

Cockroach allergen-like protein is a nitrile-specifier protein (NPS) with a detoxifying function localized in the midgut microvillar part of the insect.⁷ During digestion, CAL is released into the gut lumen, cut into fragments by trypsin-like enzymes and secreted in the stools. The most similar NPS to CAL is Bla g1 (37.6% identity, <https://www.uniprot.org/blast>), one of the major allergens of *B. germanica*. Bla g1 is labile to heat,⁸ and its genes consist of a duplex that is multiplied to create a repeated motif. A great genetic variability, in terms of the number of repeats (4-14), has been reported. The molecular weight of this allergen varies from 6 to 37 KDa, because of the action of the trypsin-like enzymes in the gut, and it is therefore considered as an 'isoallergen'.⁹ The sensitization in our 2 patients could have occurred by inhalation/contact with stools, as happens to patients allergic to cockroaches and house dust mites.

In the light of the clinical history and the results of the SPTs and specific IgEs, we can assume a primary sensitization to TM

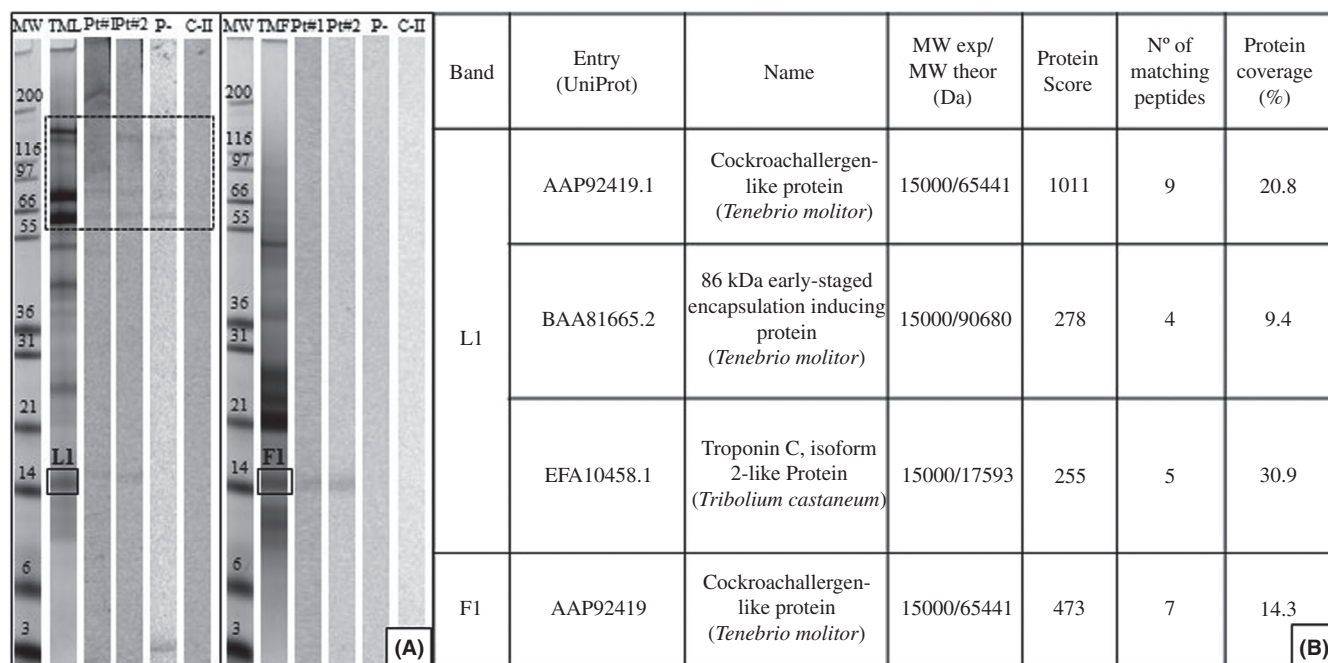


FIGURE 2 Panel A: TML and TMF protein immunoblotting with Pt#1 and Pt#2 sera. MW: molecular weight; P-: negative patient's sera; C-II: negative control without serum; the identified bands are in the continuous box; the aspecific bands are in the dotted box. Panel B: L1 and F1 protein bands identification

in our subjects, given the lack of sensitization to food allergens (crustaceans) and inhalants (mites). We consider that CAL could be the allergen that was responsible for the respiratory and food allergy in our patients, since it is present in both body and insect faeces and its heat lability could explain the type of food allergic reaction (OAS) that occurred. Moreover, the ability of patients to tolerate other edible insects and crustaceans makes the involvement of such panallergens as TnC and ESE less likely. However, we cannot exclude a possible involvement of these proteins in sensitization, perhaps after mealworm intake. Interestingly, the proteins we identified are different from those identified by Broekman and coworkers², probably because different routes of sensitization are involved in TM food allergies.

In conclusion, our patients probably developed allergy to TML because of inhalation/contact with faeces. The allergen candidate considered most responsible for the observed food allergic reaction could be CAL, which, to the best of our knowledge, has never before been implicated in allergy to TM.

CONFLICT OF INTEREST

All the authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Laura Cavallarin, Giovanni Rolla and Maria Gabriella Giuffrida conceived and designed the research; Veronica Giorgis performed SPT, and Monica Boita performed BAT; Cristina Lamberti and Stefano

Nebbia designed and performed the proteomic experiments; Marcello Manfredi and Emilio Marengo performed LC-MS/MS protein identification; Achille Schiavone contributed to biochemical considerations on insect metabolism; Veronica Giorgis, Luisa Brussino and Giovanni Rolla drew clinical consideration and conclusion; Maria Gabriella Giuffrida, Stefano Nebbia, Cristina Lamberti and Enrica Pessione wrote the first draft of the manuscript. All authors read and approved the manuscript.

ORCID

Cristina Lamberti  <https://orcid.org/0000-0003-2052-5229>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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METHODS

***Tenebrio molitor* larva and fecal extract preparation**

The *tenebrio molitor* larvae (TML) were purchased from Gaobeidian Shannong Biology CO., LTD (Gaobeidian, Hebei province, China). One gram of TML and the relative feces (TMF) were extracted for 3 hours with 5 ml of 0.1M PBS pH 7.4 with sonication each 30 min. After centrifugation at 16300xg, for 30 min at 4°C, the supernatant was collected and again centrifuged. After centrifugation, the supernatant was subsequently cleaned using a 2-D Cleanup Kit (Biorad, Hercules, USA) and the protein concentration was determined by means of a 2-D Quant-Kit (GE, Boston, USA).

Basophil Activation Test (BAT)

BAT was performed according to a previously reported technique^{E1}. Briefly, endotoxin-free heparinized whole-blood samples were obtained from the allergic patients and from a healthy control. Cells were challenged with 100 µl of TML, a TMF protein extract and an anti-IgE (10µg/ml; clone G7-18; BD Bioscience, USA) for 20 min at 37°C in a water bath. fMLP (0.5µg/ml; Sigma Aldrich, Italy) and Tyrode (Sigma Aldrich), 20µM HEPES, 7.5% NaHCO₃, pH 7.4 were used as positive and negative controls, respectively.

Basophils were stained with 5 µl of anti-human CD3 Pacific Blue (Beckman Coulter, USA), 10 µl of anti-human CD63-FITC (clone H5C6; BD Biosciences), 10 µl of anti-human CD203c-APC (clone NP4D6; BioLegend) and 10 µl of monoclonal anti-human CD294 (CRTH2) PE (Beckman Coulter, USA) for 15 min on RT. Flow-cytometric analyses were performed in a NAVIOS flow cytometer (Beckman Coulter, USA). We gated on physical parameters forward (FS) and side (SS) scatter to exclude debris. We then gated on CRTH2 (CD294) positive/CD3 negative cells to isolate basophils.

Immunoreactivity to *Tenebrio molitor* larvae and stools

SDS-PAGE: 5 micrograms of TML and TMF protein extracts were diluted in an LDS Sample Buffer (Invitrogen, Life Technologies Ltd., Paisley, UK) under reducing conditions (with 2% of a NuPAGE Sample Reducing Agent) and separated with 10% NuPAGE mini gels with an MES Running Buffer (Invitrogen), according to the manufacturer's protocol. The gels were then fixed for 2 hours in 30% ethanol and 10% orthophosphoric acid, and were stained in Colloidal Coomassie Blue^{E2}

Immunoblotting: The proteins were electro-transferred after SDS-PAGE into Nitrocellulose Membrane (0.2 µm) with an XCell II Blot Module with a Transfer buffer (Invitrogen) to which 10% methanol (v/v) had been added. The membranes were blocked with TBS containing 0.3% Tween 20 for 30 min and incubated o.n. at 4°C with the patients' sera diluted 1:10 in the incubation buffer (TBS, 0.05% Tween 20, 0.05% vegetal gelatin). After removal of the sera, the membranes were washed three times with a washing solution (TBS, 0.05% Tween 20) for 10 min each time and incubated for 1 hour at RT with an anti-Human IgE antibody (SeraCare Life Sciences Inc., Milford, Massachusetts), diluted 1:5000 in the incubation buffer. After removal of the secondary antibody, the membranes were again washed three times with the washing solution. Immunoblots were developed with an Alkaline Phosphatase Substrate Kit (Bio-Rad).

Protein identification by means of Mass Spectrometry analysis

Enzymatic in gel digestion: Bands were reduced in 10mM DTT/50 mM NH₄HCO₃ for 45 minutes at 56°C, and alkylated in 55mM IAA/50 mM NH₄HCO₃ for 30 minutes in the dark, at RT. The bands were washed with water, and destained with 50%ACN/50 mM NH₄HCO₃ (twice), pure ACN and again with 50%ACN/50 mM NH₄HCO₃. Samples were dried in an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany) and digested o.n. at 37°C

under shaking with modified porcine trypsin (Promega, Madison, Wisconsin) at 75 ng/μl of 25 mM NH₄HCO₃/10% formic acid.

Mass Spectrometry Analysis: The peptide mixtures were desalted in a Discovery® DSC-18 solid phase extraction (SPE) 96-well Plate (25 mg/well) (Sigma-Aldrich Inc., St. Louis, MO) prior to the mass spectrometry analysis. The LC-MS/MS analyses were performed by means of a micro-LC system by Eksigent Technologies, (Dublin, California). The stationary phase was a Halo Fused C18 column (0.5 x 100 mm, 2.7 μm; Eksigent Technologies). The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluted at a flow-rate of 15.0 μL/min and at an increasing concentration of solvent B from 2% to 40% over 30 minutes. The injection volume was 4.0 μL. The oven temperature was set at 40 °C. The LC system was interfaced with a 5600+ TripleTOF™ system (AB Sciex, Concord, Canada) equipped with a DuoSpray™ Ion Source and a CDS (Calibrant Delivery System). The mass spectrometer was run in data dependent acquisition mode (DDA)^{E3,E4}.

Protein database search: The DDA files were searched using Mascot v. 2.4 (Matrix Science Inc., Boston) using the NCBI Coleoptera database. Trypsin was specified as a digestion enzyme with three missed cleavages. The following parameters were set for the searches: an S-carbamidomethyl derivative on cysteine as a fixed modification and oxidation on methionine, Acetyl (N-term) and Met-loss (Protein N-term M) as variable modifications. Peptide mass tolerance was set at 50 ppm and MS/MS tolerance was set at 0.1 Da. The charges of the peptides (on a monoisotopic mass) were set at 2+, 3+ and 4+. Only proteins with at least three peptides and with a peptide score > peptide identity were considered for identification purposes.

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