

ORIGINAL ARTICLE

Immune response to mosquito allergens is associated with cross-reactive bee venom components: the importance of cross-reactive carbohydrate determinants (CCDs) in the Bee-Mosquito Syndrome

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Summary

Background. Mosquitoes are a family of the order Diptera. Throughout the world, there are two groups able to suck human blood and transmit diseases: the anophelines and the culicines comprise several genera such as Culex, Aedes and Aedes communis (Ac). Previous study has demonstrated a significant relationship between Ac sensitization and either extract or single bee venom components suggesting a “bee-mosquito syndrome” occurrence. The aim of the study was to identify bands of cross reactivity between the extracts of Mosquito and Bee venom, with IgE positive sera.

Methods. Serum from 21 different Mosquito and A. mellifera allergic individuals were combined with extracts of Mosquito (in house) and A. mellifera. SDS-PAGE and (IB) were carried out with/without CCD inhibitor.

Results. The data without CCD inhibitor suggests IgE binding to common bands between Culex male, Culex female and Aedes of approx ~21kDa, ~35kDa, ~40kDa and ~55kDa. Whilst IgE binding has been observed to protein bands from the A. mellifera extract, these bands do not align with any present in the mosquito extracts. Data with CCD inhibitor results in differences in IgE binding. Bands that were previously observed in the A. mellifera venom sample at 40 and 90 kDa, disappeared. No major differences in banding pattern were observed for the mosquito samples, although the blot appears cleaner.

Conclusions. IB data suggests that the use of CCD inhibitor prevents binding of IgE from mosquito allergic patients to multiple bands from A. mellifera venom. It may be inferred that bands

originally present at 40kDa and 90 kDa may have been due to a CCD interaction. Two bands remain present in the *A. mellifera* sample between 15-20 kDa, however do not align with any bands present in the mosquito extracts. Further experiments must be done to determine whether the reactive bands from *A. mellifera* are unrelated proteins or whether the proteins are related homologues of varying MW.

Key words

Insect allergy- bee-mosquito syndrome; CCD; molecular diagnosis, immunoblot; CCD-inhibitor

Impact statement

Our results highlight the importance of the use of CCD inhibitor for an accurate diagnosis in the "bee-mosquito syndrome". Further studies are urgent to identify new specific allergenic proteins.

Introduction

Mosquitoes are a family of insects of the order Diptera and are responsible for most insect bites worldwide (1). The local reaction usually produced after a mosquito bite can be sometimes severe and systemic reactions have rarely been reported (2).

For some individuals, a large local reaction (wheal > 5 mm) occurs within minutes to hours. These individuals may be diagnosed with a mosquito allergy (2) but unfortunately, allergic reactions to mosquito bites are underestimated due to the lack of reliable diagnostic tools.

Both "species-specific" and cross-reactive allergenic molecules have recently been described mainly in the saliva of yellow fever mosquito (*Aedes aegypti*), Asian tiger mosquito (*Aedes albopictus*), *Aedes vexans*, and *Culex quinquefasciatus* (3–5). Few studies have been conducted on *Aedes communis* (Ac) (6,7) and no Ac molecule has been registered to date in the WHO/IUIS database (8).

Recently, it has been described that in individuals with severe local reactions following mosquito bites, the immune response to mosquito allergens was associated with both species-specific (Api m 1, Api m 3, and Api m 10), and cross-reactive (Api m 2 and Api m 5) bee venom components, suggesting the "Bee- Mosquito syndrome" (9)

Unfortunately, mosquitoes and horseflies' whole-body extracts for in vitro and in vivo diagnosis, are limited. The sensitivity and specificity of these extracts are very low due to the scarce presence of relevant

salivary gland proteins and because the IgE binding is often to non-salivary proteins (e.g., tropomyosin) or to the presence of cross-reactive carbohydrate determinants (CCD)-sIgE (10). Several studies have demonstrated that prevalence rates for positive CCD-sIgE reactivity are approximately 20–37% in allergic patients with grass pollen exposure and Hymenoptera stings (11 - 15)

The risk of CCD interference that compromises quantitative IgE results can be mitigated by the addition of a soluble CCD inhibitor to positive CCD-sIgE containing sera or alternatively using a non-cellulose-based sIgE assay. (11)

Hence, it has been hypothesized that the presence of sIgE to CCD could have a role in the Bee-Mosquito syndrome.

Material and Methods

First Experimental procedure

Extract Preparation

100 of each mosquito species provided by Experimental Zooprophyllactic Institute of Sicily were ground using a disposable spatula and thoroughly mixed by hand. Protein was extracted in 2x phosphate buffered saline + 2mM phenylmethylsulfonyl fluoride at 4°C. Briefly, samples were mixed by vortex for 30 seconds and sonicated for 15 minutes. Samples were centrifugated at 8000g for 30 minutes at 4°C. The supernatant was collected and stored at -20°C. 5mg *A. mellifera* extract (Citeq) was reconstituted in 5ml 0.9%NaCl, pH 7.4. The reconstituted extract was stored at -20°C. Extracts of Mosquito and *A. mellifera* were quantified for total protein content using a BCA protein kit (Pierce 23225).

Serum Pool Generation

Serums from 21 outpatient mosquito-allergic patients were collected at Istituto Dermopatico dell'Immacolata - IRCCS, Rome and Buccheri La Ferla Fatebenefratelli Hospital, Palermo. All patients reported immediate large local reactions characterized by hives and cutaneous angioedema lasting several hours after mosquito bites. They were all positive to sIgE to mosquitoes, *A.*

mellifera, Api m1, Api m 2, Api m 3, Api m 5, Api m 10 and negative to sIgE to CCD (bromelain N-glycan MUXF3, ImmunoCap Phadia Thermo Fisher, Italy). The pooled sera were aliquoted and stored at -20°C, then at -80°C for long-term storage. The study was approved by the ethics committee Palermo 1 number 1/2020 and approved informed consent was obtained from all patients.

SDS-PAGE

Samples were run on polyacrylamide gels by Indoor Biotechnologies Ltd, according to their standard procedures. Briefly, samples were prepared by mixing with Laemlli sample loading buffer with 0.1M DTT and heated for 5 minutes at 100°C. Samples were loaded at 15 µg (according to concentration determined in 2.1) per well into 4–20% Mini-PROTEAN® TGXTM Precast Protein Gels (BioRad) and run at 300V for 20 minutes. Gels were taken forward for Coomassie staining by incubating with Instant Blue protein stain for a minimum of 1 hour or Immunoblotting.

Immunoblot

Western Blots were carried out by Indoor Biotechnologies Ltd using an adapted version to account for the use of human sera and an alternative secondary antibody. Blocking, antibody wash, diluent solutions, and chemiluminescent substrate were obtained and prepared using reagents from the Western Breeze blot detection kit (Invitrogen WB7104). Proteins were transferred to PVDF membranes using Trans-Blot Turbo Transfer Pack, mini gel, 0.2 µm PVDF (Bio-Rad, Cat No. 1704156) according to manufacturer instruction. Membranes were blocked following transfer for 1 hour at room temperature and washed with distilled water. Pooled sera were applied to the blot at a 1:5 dilution in antibody diluent and incubated overnight at 4°C. A negative control assay was also incubated overnight with antibody diluent at 4°C without sera. Membranes were washed using antibody wash solution before the addition of Goat anti-Human IgE-Alkaline phosphatase conjugate (BioRad STAR147A) at 1:5000 in antibody diluent and incubated for 1 hour at room temperature before washing with antibody wash solution and incubation for 5 minutes with a chemiluminescent substrate.

When typically running an immunoblot, it was used Streptactin-Alkaline phosphatase conjugate to

detect molecular weight markers in chemiluminescent western blots. During optimization, it was discovered that Streptactin-Alkaline phosphatase conjugate was bound to samples and caused interference. Therefore, this reagent was left out, and western blots required two reads – one to detect samples by chemiluminescent read and a second colorimetric read to detect standards.

Results

Total protein content

Samples were quantified for total protein content by BCA protein kit (2.1). The results of the analyses are summarized below in Table 1.

SDS-PAGE

Mosquito and bee venom extracts prepared in 2.1 were run on SDS-PAGE (2.3), with data presented in Figure 1.

Immunoblot

Mosquito and bee venom extracts prepared in 2.1 were subject to immunoblotting (2.4) with pooled sera from 21 mosquito-allergic donors. Data is presented below in Figure 2. A negative control blot run without the addition of primary antibody (sera) can be found in the appendices – Figure 4.

The immunoblot experiments show that the IgE from the mosquito allergic sera pool binds to multiple protein bands present in *Culex* male, *Culex* female, *Aedes* species mosquitos and to *A. mellifera* venom extract (Figure 3)

A follow-on experiment was performed to assess the use of CCD-Inhibitor and whether this would prevent IgE binding in immunoblot.

Second Experimental procedure

Sample preparation

Mosquito and bee venom extracts, as well as pooled sera were prepared as described in the first experimental procedure. These were taken forward for use in SDS-PAGE and Immunoblot

procedures.

Incubation of Sera with CCD-Inhibitor

Pooled sera were incubated with CCD-Inhibitor (R-Biopharm, Cat No. ZA0601) according to manufacturer instructions. Briefly, 2ml of pooled sera was incubated with 50µl reconstituted CCD-Inhibitor and incubated for 1 hour at room temperature in an orbital incubator at 65rpm. CCD-Inhibited sera were used immediately after 1 hour of incubation for overnight incubation in immunoblot.

Discussion

SDS-PAGE

Mosquito and bee venom extracts prepared in the first experiment were run on SDS-PAGE, with data presented in Fig.1.

Immunoblot

Mosquito and bee venom extracts prepared in the first experiment were subject to immunoblotting with pooled sera from 21 mosquito allergic donors. Sera was incubated with CCD-Inhibitor prior to use. Data is presented in Fig. 5

The immunoblot experiments (Fig. 6) demonstrate that the use of a CCD-Inhibitor results in differences in IgE binding. Specifically, bands that were previously observed in the *Apis mellifera* venom sample without use of CCD-Inhibitor at 40kDa and 90kDa are no longer observed when CCD-Inhibitor is used. Two bands of the *Apis mellifera* venom remain present between 15-20kDa. We have speculated that the band of a MW between 15 and 20 kDa in *A. mellifera* venom could be The phospholipase A2 and the band of about 40 kDa could refer to the hyaluronidase. The latter has been identified as the most important allergens displaying CCDs and it could then be presumed that this is why the CCD-inhibited sera don't bind it.

No major differences in banding pattern were observed for the mosquito samples with or without CCD-Inhibitor, although the blot appears cleaner with less background when CCD-Inhibitor is used.

Pooled sera from patients with negative CCD markers (MUXF) show differences in binding allergens before and after incubation with CCD-inhibitors because CCD markers alone are not a sufficient tool

in identifying in vitro CCD interference. It is well known that approximately 30% of the allergic population sera contain sIgE to CCD thus, component resolved diagnosis (CRD) using recombinant allergens with no apparent glycosylation, has been recommended to reduce the risk of inaccurate results. However, a recent study has shown that some commercial assays have a polymerized cellulose matrix used to bind allergenic proteins contains CCD epitopes that are recognizable by IgE antibodies. This means that the sIgE to CCD of a patient tested against a “CCD-free recombinant protein,” may recognize N-glycans present on the cellulose matrix; which would result in an increased rate of false-positive results, and at a minimum, reduce confidence in the accuracy of results generated on these cellulose based assays. Many clinicians are unaware of this issue therefore the interpretation of recombinant CRD results may lead to an incorrect diagnosis if the patient sera contain levels of sIgE to CCD. (16,17)

Immunoblot data suggests that the use of CCD-Inhibitor prevents binding of IgE mosquito allergic individuals to multiple bands from *Apis mellifera* venom. It may be inferred that bands originally present at 40kDa and 90kDa therefore, may have been due to a CCD interaction. Two bands remain present in the *Apis mellifera* sample between 15-20kDa, however these do not align with any bands present in the mosquito extracts.

Future experiments are necessary to determine whether the reactive bands from *Apis mellifera* are unrelated proteins or whether the proteins are related homologues of varying molecular weight.

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Contributions: CGU: conceptualization, methodology, project administration, resources, supervision, writing-original draft, review & editing-CDA, CDS, VB, MB: data curation, investigation, validation, visualization ES, AT: conceptualization, resources, IB: resources, formal analysis

Conflict of interests: The authors declare that they have no conflict of interest.

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Figure 1. SDS PAGE of mosquito and bee extracts. Samples run as follows: 1. Marker, 2. Culex Sp Male, 3. Culex Sp Female, 4. Aedes sp, 5. Apis venon extract, 6. Marker

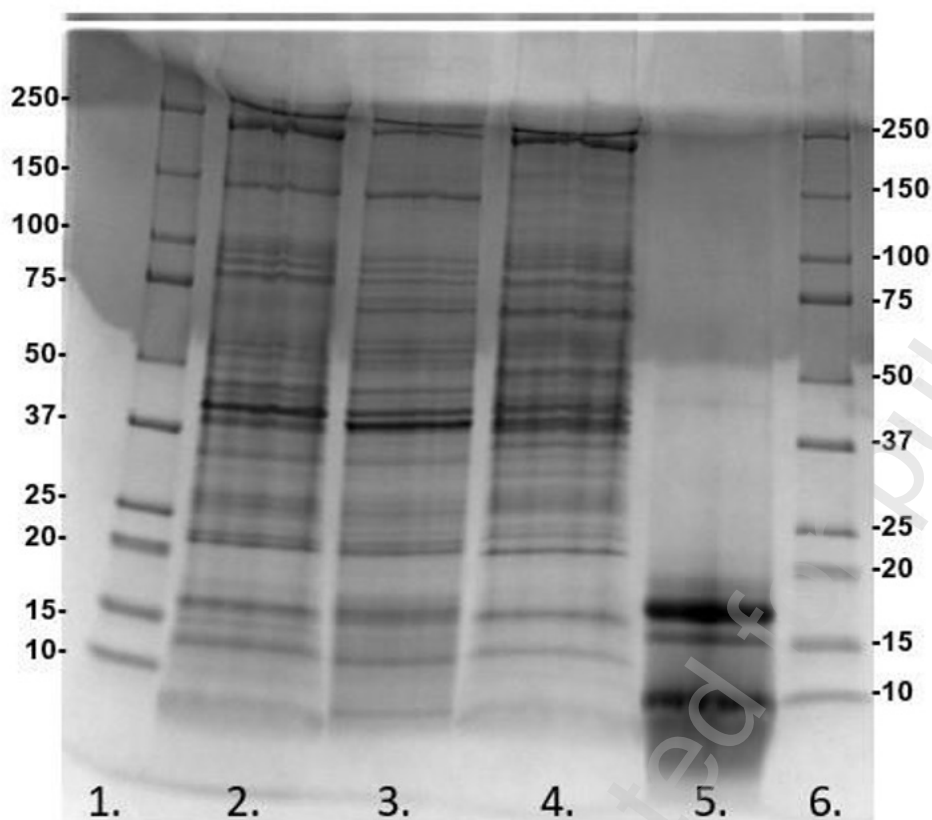


Figure 2. Immunoblot of mosquito and bee extracts. Samples run as follows: 1. Marker, 2. Culex Sp. Male, 3. Culex Sp. Female, 4. Aedes Sp., 5. Apis venom extract, 6. Marker. Note a black box surrounds samples on the blot. The image contained within the black box are obtained by chemiluminescent read, whereas all image outside of the read box are obtained by colorimetric read. The two images were overlayed to create Fig. 2 to display the molecular weight marker and samples on the same image. Molecular weight marker could not be visualized by chemiluminescent read and had to be visualized in a colorimetric read. Both individual images can be found in appendice 01. A negative control blot run without the addition of primary antibody (sera) can be found in the appendices Figure 4.

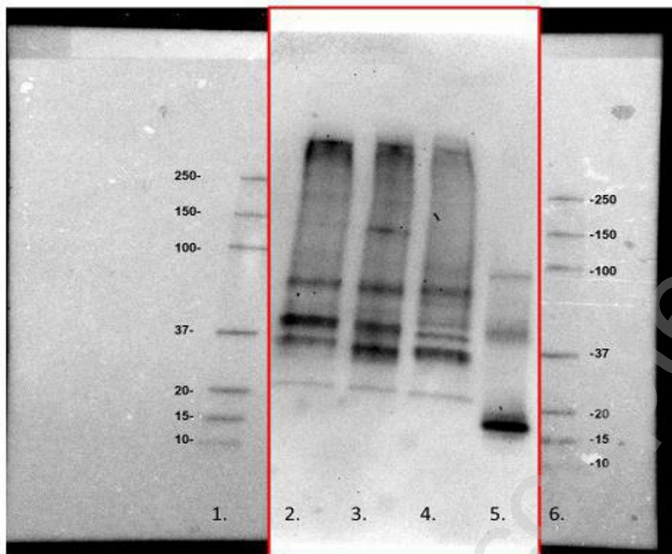


Figure 3. Side by side comparison of SDS-PAGE and Immunoblot data from mosquito and bee extracts. Samples run as follows: 1. Marker, 2. Culex Sp. Male, 3. Culex Sp. Female, 4. Aedes Sp., 5. Apis venom extract, 6. Marker. See figures 1 and 2 for individual descriptions. A negative control blot that runs without the addition of primary antibody (sera) can be found in the appendices Figure 4.

Figure 3

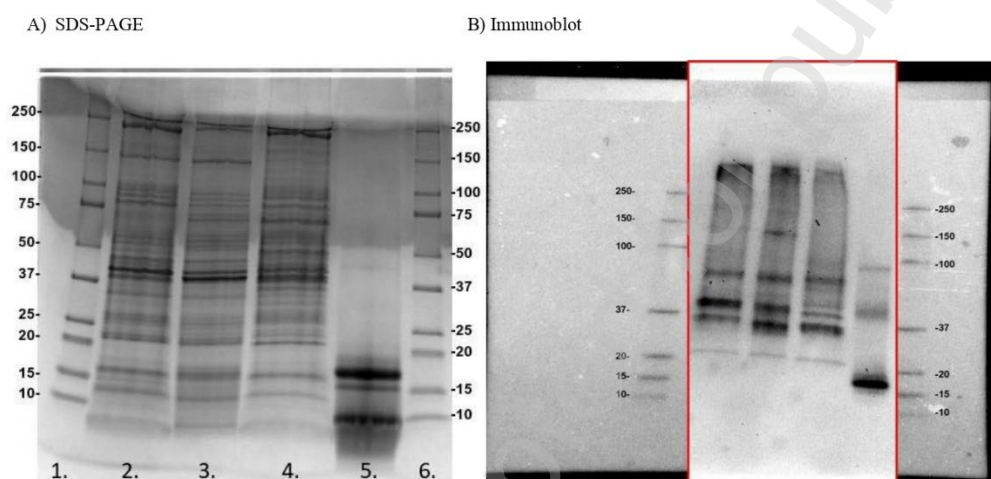


Figure 4. Immunoblot negative control. Immunoblot without the addition of human sera (primary antibody). Samples run as follows: 1. Marker, 2. Culex Sp Male, 3. Culex Sp Female, 4. Aedes Sp, 5. Apis venom extract. Note a black box surrounds samples on the blot. Image contained within the black box are obtained by chemiluminescent read, whereas all image outside of the red box are obtained by colorimetric read. The two images were overlaid to create Fig 4 to display the molecular weight marker and samples on the same image. Molecular weight marker could not be visualised by chemiluminescent read and had to be visualised in a colorimetric read. Both individual images can be found in Figure 02 of appendices.

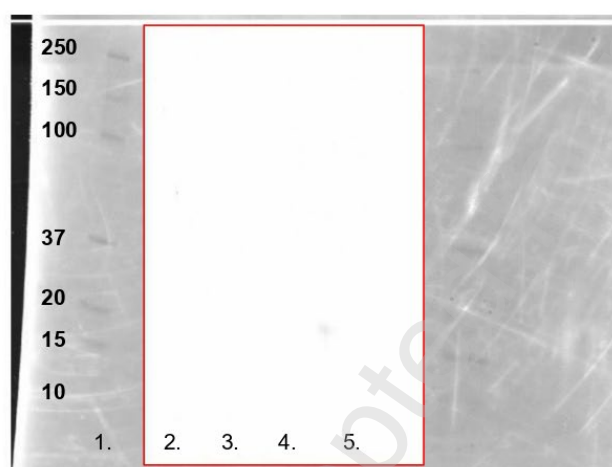


Figure 5. Side by side comparison of Immunoblot data from mosquito and bee extracts with and without CCD-Inhibited sera. A) Without CCD-Inhibitor. B) With CCD-Inhibitor. Samples run as: 1. Marker, 2. Culex Sp Male, 3. Culex Sp Female, 4. Aedes Sp., 5. Apis mellifera venom extract, 6. Marker. A negative control blot without the addition of primary antibody sera can be found in previous experiment.

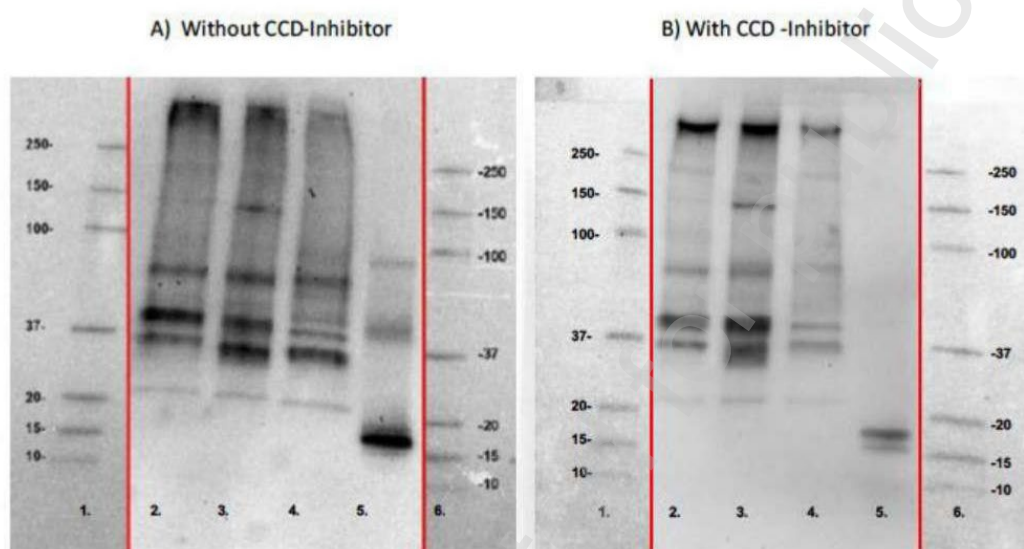
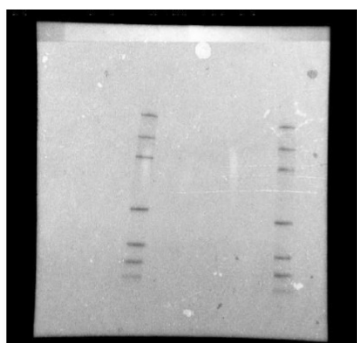


Table 1. Total protein concentration of mosquito and bee venom extracts.

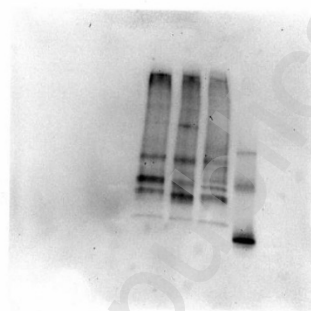
Sample	Total protein concentration (mg/ml)
Culex mosquito male	1.1
Culex mosquito female	2.1
Aedes mosquito	1.0
Apis mellifera extract	3.3

Appendix 001

A) Colorimetric

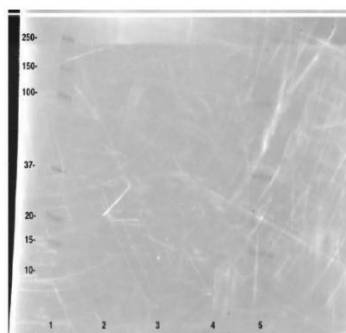


B) Chemiluminescent



Appendix 002

A) Colorimetric



B) Chemiluminescent

