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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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Abstract: Background: Mosquitoes are a family of the order Diptera, which includes more than 3500 species. Culex, Aedes and Aedes communis (Ac) are the most well-known species. Previous study has demonstrated a significant relationship between Ac sensitization and either extract or single bee venom components suggesting a "bee-mosquito syndrome" occurrence. Several studies have demonstrated that prevalence rates for positive CCD-sIgE reactivity in allergic patients with grass pollen exposure and Hymenoptera stings. The risk of CCD interference that compromises quantitative IgE results can be mitigated by the addition of a soluble CCD Inhibitor. The aim of the study was to identify bands of cross reactivity between the extracts of Mosquito and Bee venom, with IgE positive sera using a CCD Inhibitor. Methods: Serum from 21 different Mosquito and Ape melifera allergic individuals were combined with extracts of Mosquito (in house) and Apis melifera. SDS-PAGE *and Immunoblot (IB) were carried out.* Results: bands that were previously observed in the *Apis melifera* 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 melifera* venom remain present between 15-20kDa. **Conclusions:**Immunoblot data suggests that the use of CCD-Inhibitor prevents binding of IgE mosquito allergic individuals to multiple bands from *Apis melifera* venom. Future experiments are necessary to determine whether the reactive bands from *Apis melifera* are unrelated proteins or whether the proteins are related homologues of varying molecular weight.

Keywords: mosquito allergy; bee-mosquito syndrome; CCD-Inhibitor

1. 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*),

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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 hypnotized that the presence of sIgE to CCD could have a role in the Bee-Mosquito syndrome.

2. Material and Methods

First Experimental procedure

2.1. Extract Preparation

100 of each mosquito species provided by Experimental Zooprophylactic 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 4oC. Briefly, samples were mixed by vortex for 30 seconds and sonicated for 15 minutes. Samples were centrifugated at 8000g for 30 minutes at 4oC. The supernatant was collected and stored at -20oC. 5mg A. *mellifera* extract (Citeq) was reconstituted in 5ml 0.9%NaCl, pH 7.4. The reconstituted extract was stored at -20oC.

Extracts of Mosquito and A. *mellifera* were quantified for total protein content using a BCA protein kit (Pierce 23225).

2.2. Serum Pool Generation

Serums from 21 different mosquito-allergic patients were combined. 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 -20oC, then at -80oC for long-term storage.

2.3. SDS-PAGE

Samples were run on polyacrylamide gels according to Indoor Biotechnologies Ltd SOP IBL LAB SOP-021. Briefly, samples were prepared by mixing with Laemlli sample loading buffer with 0.1M DTT and heated for 5 minutes at 100oC. Samples were loaded at 15µg (according to concentration determined in 2.1) per well into 4–20% Mini-PROTEAN® TGX™ 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.

2.4. Immunoblot

Western Blots were carried out using an adapted version of Indoor Biotechnologies Ltd SOP IBL LAB SOP-023 to account for the use of human sera and an alternative secondary antibody. Blocking,

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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, 0.2µm 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.

3. Results

3.1. 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.

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

Sample	Total protein concentration (mg/ml)
Culex mosquito (Male)	1.1
Culex mosquito (Female)	2.1
Aedes Mosqutio	1.0
Apis melifera extract	3.3

3.2. SDS-PAGE

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

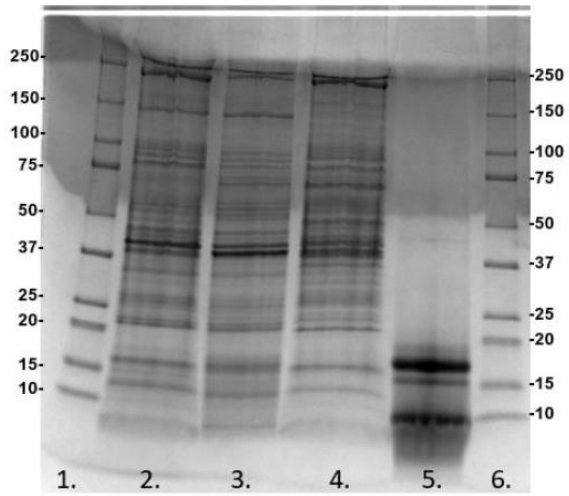

Figure 1. SDS-PAGE of mosquito and bee extracts. *Samples run as follows: 1. Marker, 2. Culex Sp. Male, 3. Culex Sp. Female, 4. Aedes Sp. 5. Apis venom extract. 6. Marker.*

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3.3. 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.

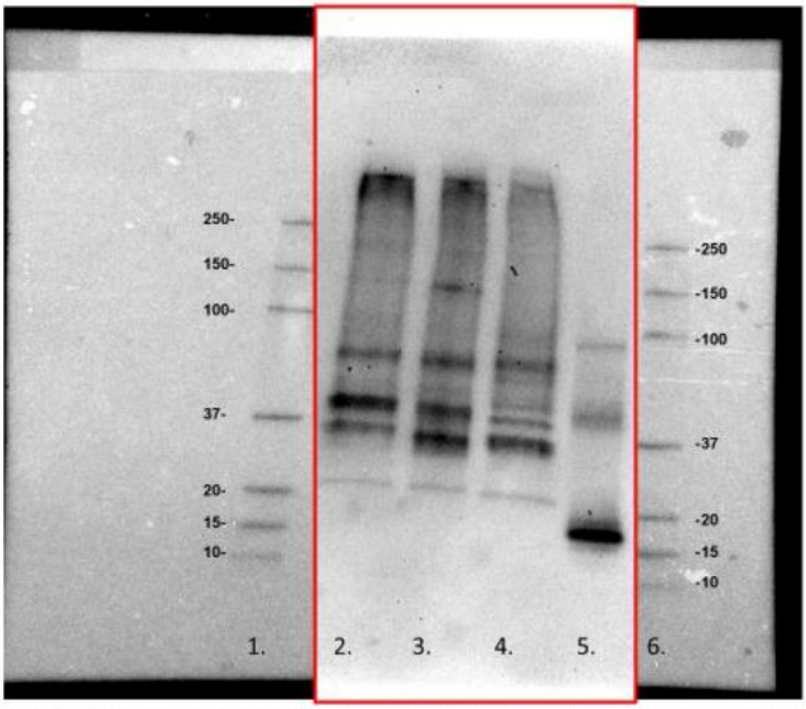

Figure 2. Immunoblot of mosquito and bee extracts. *Samples run as follows: 1. Marker, 2. Culex Sp. Male, 3. Culex Sp. Female, 4. Aedes Sp. 5. Apis venom extract. 6. Marker. Note a red box surrounds samples on the blot. Image contained within the red box are obtained by chemiluminescent read, whereas all image outside of the red box are obtained by colorimetric read. The two images were overlayed to create Figure 2 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 5. of appendices. A negative control blot run without the addition of primary antibody (sera) can be found in the appendices – Figure 4.*

Figure 3. The SDS-PAGE and Immunoblot data were aligned as below.

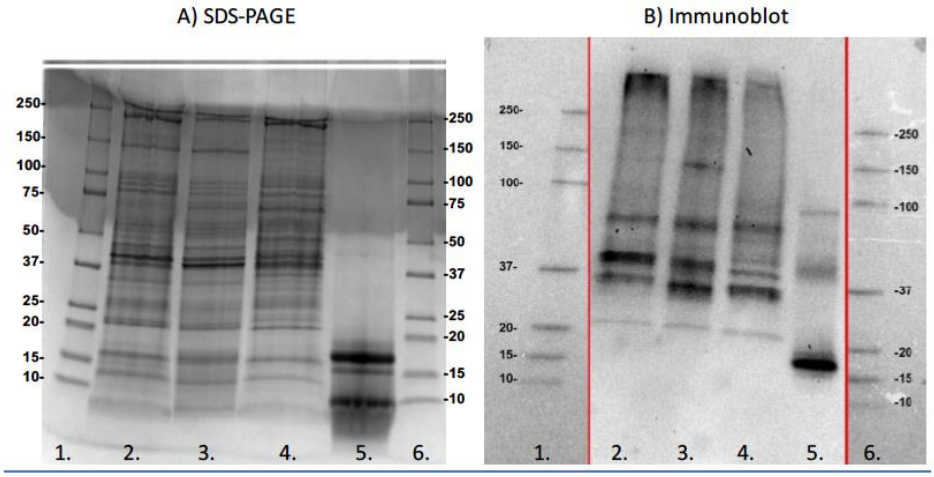

Figure 3. Side by side comparison of SDS-PAGE and Immunoblot data for mosquito and bee extracts. *Samples run as follows: 1. Marker, 2. Culex Sp. Male, 3. Culex Sp. Female, 4. Aedes Sp. 5. Apis venom extract. 6. Marker. See Figures 1. and 2. for individual descriptions. A negative control blot run without 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)

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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

4.1. Sample Preparation

Mosquito and be 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.

4.2. 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.

4.3. Results

4.4. SDS-PAGE

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

4.5. 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 Figure 2.

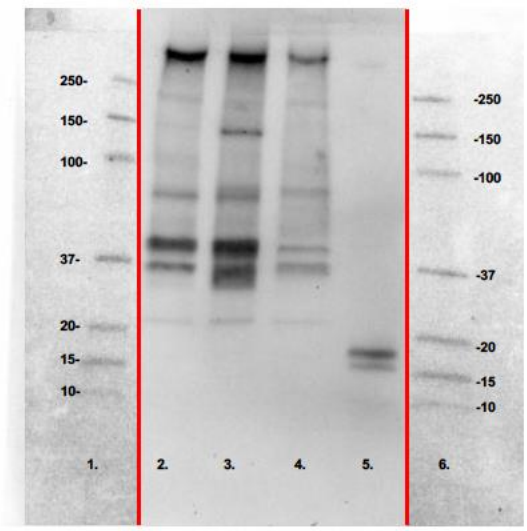

Figure 2. Immunoblot of mosquito and bee extracts with CCD-Inhibitor. Samples run as follows: 1. Marker, 2. Culex Sp. Male, 3. Culex Sp. Female, 4. Aedes Sp. 5. Apis meliera venom extract. 6. Marker. Note a red box surrounds samples on the blot. Image contained within the red box was obtained by chemiluminescent read, whereas all image outside of the red box was obtained by colorimetric read. The two images were overlayed to create Figure 2 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 4. of appendices. Control blots were run without the addition of primary antibody (sera) and CCD-Inhibitor and can be found in CR20-0012 report.

Figure 2 was aligned with the immunoblot carried out in the first experiment without CCD-Inhibitor. Aligned blots are presented in Figure 3.

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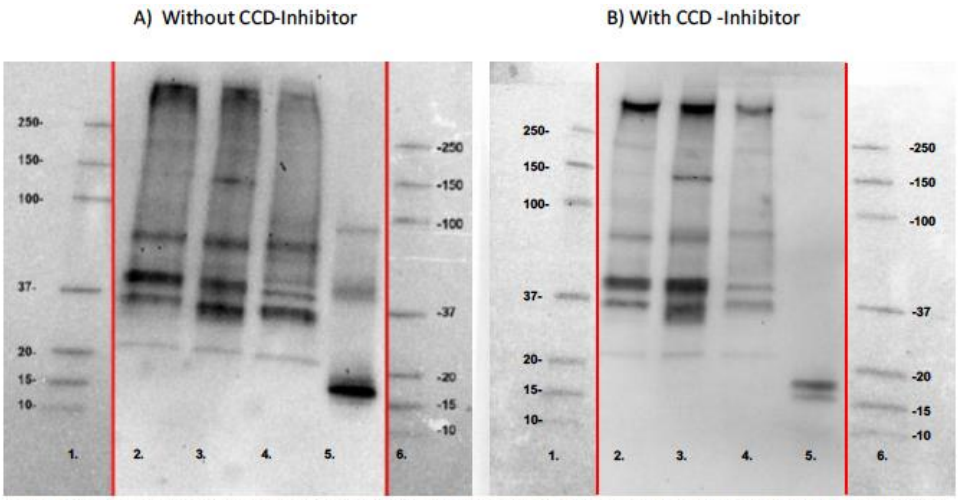

Figure 3. Side by side comparison of Immunoblot data for mosquito and bee extracts with and without CCD-Inhibited Sera. A) Without CCD-Inhibitor. B) With CCD-Inhibitor. Samples run as follows for both Immunoblots: 1. Marker, 2. Culex Sp. Male, 3. Culex Sp. Female, 4. Aedes Sp. 5. Apis melifera venom extract. 6. Marker. A negative control blot run without the addition of primary antibody (sera) can be found in CR20-0012 report.

The immunoblot experiments (Figure 3) demonstrate that the use of a CCD-Inhibitor results in differences in IgE binding. Specifically, bands that were previously observed in the *Apis melifera* 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 melifera* venom remain present between 15-20kDa.

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.

5. Conclusions

Immunoblot data suggests that the use of CCD-Inhibitor prevents binding of IgE mosquito allergic individuals to multiple bands from *Apis melifera* 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 melifera* 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 melifera* are unrelated proteins or whether the proteins are related homologues of varying molecular weight.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: title; Table S1: title; Video S1: title.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflicts of interest.

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