ARQUIVOS DE ASMA, ALERGIA E IMUNOLOGIA

Original Article

Immunological analysis of allergenic cross-reactivity between *Cheyletus malaccensis* and *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* and *Blomia tropicalis*

Análise imunológica da reatividade cruzada alergênica entre Cheyletus malaccensis *e* Dermatophagoides farinae*,* Dermatophagoides pteronyssinus *e* Blomia tropicalis

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ABSTRACT

Objective: The mite *Cheyletus malaccensis* is cited in the literature as a predator of other mite species. Little is known about its protein composition, and few studies have evaluated its ability to trigger atopic respiratory allergic reactions. The present study aims to investigate the protein profile fingerprint present in *Cheyletus malaccensis* extract and to evaluate its immunologic reactivity in the presence of specific immunoglobulins (IgE) from the serum of individuals diagnosed with allergy to the mites *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* and *Blomia tropicalis*. These three species carry proteins responsible for the most cases of atopic respiratory allergies, hence the interest in comparing them to *Cheyletus malaccensis*. **Methods:** Samples of aspirated dust containing *Cheyletus malaccensis* were collected from households in the city of Rio de Janeiro, Brazil. From the collected mass of this mite, extracts were prepared for analysis. Proteins present in the extracts were identified by electrophoresis under denaturing conditions. **Results:** Proteins with a molecular mass of 24 kDa, 26 kDa, 12 kDa, 45 kDa and 70 kDa were visualized. The immunoblotting assay showed positive cross-reactivity for proteins of molecular mass ranging from 20 kDa to 45 kDa. These results indicate that specific links were established between IgE present in the serum of individuals allergic to the comparator mite and proteins from *Cheyletus malaccensis*. **Conclusions:** These findings are relevant for their potential clinical and immunotherapeutic applications, as well as information base for further studies.

Keywords: Mites, *Cheyletus malaccensis*, protein characterization, allergens, cross-reactivity.

RESUMO

Objetivo: O ácaro *Cheyletus malaccensis* é referido na literatura como um predador de outras espécies de ácaro. Pouco se sabe sobre sua composição proteica, e poucos estudos avaliaram sua habilidade de desencadear reações alérgicas respiratórias atópicas. O objetivo do presente estudo é investigar a impressão digital do perfil proteico presente em um extrato de *Cheyletus malaccensis* e avaliar sua reatividade imunológica na presença de imunoglobulinas (IgE) específicas do soro de indivíduos diagnosticados com alergia aos ácaros *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* e *Blomia tropicalis*. Essas três espécies carregam proteínas responsáveis pela maioria dos casos de alergias respiratórias atópicas, o que justifica o interesse em compará-las ao *Cheyletus malaccensis*. **Métodos:** Amostras de poeira aspirada contendo *Cheyletus malaccensis* foram coletadas de domicílios na cidade do Rio de Janeiro, no Brasil. A partir da massa coletada desse ácaro, extratos foram preparados para análise. As proteínas presentes nos extratos foram identificadas por eletroforese sob condições desnaturantes. **Resultados:** Proteínas com massa molecular de 24 kDa, 26 kDa, 12 kDa, 45 kDa e 70 kDa foram visualizadas. O ensaio imunoenzimático mostrou reatividade cruzada positiva para proteínas de massa molecular variando de 20 kDa a 45 kDa. Esses resultados indicam que ligações específicas foram estabelecidas entre a IgE presente no soro de indivíduos alérgicos ao ácaro usado como comparador e proteínas de *Cheyletus malaccensis*. **Conclusões:** Os achados são relevantes por seu potencial clínico e aplicações imunoterapêuticas, bem como sua base de informações para futuros estudos.

Descritores: Ácaros, *Cheyletus malaccensis*, caracterização de proteínas, alérgenos, reatividade cruzada.

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Introduction

The mite *Cheyletus malaccensis* is cited in the literature as a predator of other species of mites.[1-5] Its main habitat is humid climates. In some cases, it is found in the epidermis of cats, probably not as parasite but as predator of other mites present on the animal. Their preferred niche is hay warehouses, grain silos, and environmental dust – especially household dust, in which mites common to their diet can be found.[6]

The literature has many references to the *Cheyletidae* family of mites in relation to respiratory allergies. Homology among mites of the same and different species has been reported, such as *Dermatophagoides* sp and *Blomia tropicalis;*[7,8] allergens present in *Blomia tropicalis*, Blo t 1, present 53% sequence homology with group 1 allergens from Der p1 (*D. pteronyssinus*) and Der f 1 (*D. farinae*), and 51% homology with Erm1 (from *Euroglyphus maynei*). The literature presents cases of correlation between proteins extracted from *D. pteronyssinus* and *D. farinae* against serum antibody (IgE) from individuals sensitized to these mites.[9,10]

Similarities for proteins of molecular weight of 15 kDa were determined characterizing the allergen of group 1, Der f 2 and Der p 2 and similarity in composition and amino acid sequence. Based on these results, it can be inferred that immunotherapy against *Dermatophagoides* spp can act in terms of cross-reactivity in individuals sensitized by *B. tropicalis.*[11] Hence, the presence of homologue allergenic proteins among mites can also indicate the possibility of cross-reactivity among different species, which has been reported in several papers.

There is a shortage of scientific literature regarding the characterization of *C. malaccensis* proteins for immunotherapeutic and diagnostic use. More in-depth studies on this matter would be of interest, because the possibilities of cross-reactions with mites that are common triggers of human allergies could facilitate diagnostic tests and even lead to more efficient immunotherapies. Thus, as a predator, *C. malaccensis* may contain allergenic proteins that are present in the most prevalent mites of tropical countries, such as *D. pteronyssinus*, *D. farinae* and *B. tropicalis*. This study aims to investigate the proteins extracted from *C. malaccensis* against the serum of patients sensitized by the aforementioned mites, in hopes of contributing to future research.

Materials and methods

Taxonomic identification

Dust samples were collected from households located in the city of Rio de Janeiro, Brazil. *C. malaccensis* was isolated from surface dust mechanically aspirated with a portable vacuum cleaner (NV3600, Black & Decker) from beds, pillows, sofas, carpets, and soiled areas. All particulate material was sifted in 80-mesh sieves, and mites contained therein were isolated and identified with the aid of taxonomic reference catalogs and identification keys.[13] The main identifying characteristics of the anterior region of *C. malaccensis* (the gnathosoma) include mouthparts (chelicerae) in the form of two curved stilettos, having wide and free palps, and other peculiarities.[13]

Cultivation of mites

Samples of *C. malaccensis* were grown at room temperature (25 °C). The culture vessel was kept encased by a vessel containing water for 90 to 180 days. The mites were fed fish chow every 15 days. After the growth period, the mite mass was isolated from the debris through a 400-mesh sieve and killed with organic solvent at 40 °C. *D. farinae*, *D. pteronyssinus* and *Blomia tropicalis* extracts were obtained from Laboratório de Extratos Alergênicos Ltda. (Rio de Janeiro, Brazil).

Extraction of protein constituents

The preparation of the protein extracts followed a modified version of a procedure described elsewhere.[14] In a buffered saline solution (0.01 mol·L^{-1} TBS, pH 7.0), 10% (w/v) of the mite mass was added and homogenized in 0.1 mol·L^{-1} saline buffer using a Sonicator (Concertot) at 20 kHz frequency for 30 minutes to induce cell disruption. The pH of the extract was adjusted to 8.5 with 2.0 mol·L^{-1} NaOH solution. After homogenization, the extract was allowed to stand at 8 °C for 48 h and then centrifuged at 1500 × g for 30 minutes at 25 °C. The supernatant was filtered through a 0.22 µm pore membrane and 40% (v/v) glycerol was added. At the end of the process, the pH was adjusted to 7.0.

Determination of protein content and reducing sugars

The protein content of the extract was determined by the Lowry method following U.S. Pharmacopoeia

standards.[15] Concentrations were determined by the calibration curve using bovine serum albumin (BSA) as the standard protein (Sigma-Aldrich) in the range of 0.01 mg·mL^{-1} to 0.07 mg·mL^{-1}. For each point of the curve, 3.0 mL of 1% sodium carbonate and 0.5 mL of 0.10% copper sulfate was added and then homogenized. After standing for 10 minutes at 25 °C, 500 µL of Folin-Ciocalteu reagent (1:10) was added. The test tubes were left to stand for 30 minutes in the dark. Absorbance readings were performed on a UV-Visible Spectrophotometer (SP 1102, BEL Photonics, Brazil) at a wavelength of 625 nm. The protein content was obtained through the equation of the line, considering the correlation coefficient. The reducing sugar content of the extract sample was determined using dinitrosalicylic acid (DNS) according to the classic method with modifications.[16] A calibration curve was prepared using glucose as the standard solution in the concentration range of 0.10 to 1.0 mg·mL^{-1}. In a test tube, 1.0 mL of the sample was added to 1.0 mL of the DNS reagent. After heating to 100 °C (5 min) and cooling, 16.0 mL of $KNaC_4H_4O_6{\cdot}4H_2O$, 0.20 mol·L^{-1}, was added. The readings were performed at 540 nm using the aforementioned spectrophotometer.

Preparation of 12.5% polyacrylamide gel (SDS-PAGE)

Protein separation and characterization of *C. malaccensis* mite extract was performed by electrophoresis in denaturing conditions, as described in the literature.[17] The technique is based on the separation of the proteins by the distance covered in the gel and the molecular weight of the protein. Stacking (upper) gel for sample addition and protein organization for subsequent separation was prepared from 0.5 mL acrylamide/bis-acrylamide 30% solution (29:1), 2.5 mL Tris-HCl-SDS buffer 0.25 M/pH 6.8, 1.925 mL distilled water, 75 µL APS (ammonium persulfate) 10% w/v, and 7.5 µL TEMED (N,N,N′,N′-tetramethylethylenediamine). For the resolving (lower) gel, where proteins separation occurs, a 12.5% acrylamide concentration was achieved by addition of 4.125 mL acrylamide/bis-acrylamide 30% solution, (29:1); 5.0 mL Tris-HCl-SDS buffer 0.75 M (pH 8.8), 75 µl APS (ammonium persulfate) 10 %(w/v), 50 µL TEMED and 0.8 mL distilled water. The electrolytic solution used for the electrophoretic run was Tris-glycine 5.0 mmol·L^{-1}, pH 8.6. The applied potential for running was 150 V and 30 mA for 90 minutes. A 30.0 µL aliquot of the *C. malaccensis* extract sample (concentration in mg/mL) was added and 15.0 µL of reducing buffer containing 0.1 mol·L-1 Tris-Cl, pH 8.45, 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol and 0.04% bromophenol blue. To compare the migrations, a standard-color protein marker (Dual Xtra-Bio-Rad) with a molecular weight range of 250 kDa to 10 kDa was used with gelAnalizer10a software to determine the molecular mass (kDa) of protein constituents from *C. malaccensis* extract.

Immunoblotting

Proteins from the extracts were separated by 5-12.5% gradient gel SDS-PAGE electrophoresis and transferred to a 0.45-µm pore nitrocellulose membrane (Bio-Rad). The membrane and gel containing the allergens were sandwiched, covered with filter paper and compressed with polyurethane sponges, onto the transfer support. All material was pre-soaked in saline buffer (pH 7.2), consisting of 25 mmol·L^{-1} Tris-Base, 192 mmol·L^{-1} glycine and 20% methanol. Slow transfer conditions were applied with a constant voltage of 18 V, current 10 mA and power 70 W for 18 h at 8 °C. To confirm transfer of the proteins from the gel to the nitrocellulose membrane, a 0.2% (m/v) solution of Ponceau-S dye was added.

IgE reactivity test

For the reactivity test, we used serum of 15 individuals allergic to *D. farinae*, *D. pteronyssinus* and *B. tropicalis*. The membrane containing the proteins was initially blocked with bovine serum albumin solution (0.5%) in saline buffer (TBS) for 1 h under gentle agitation, to avoid nonspecific binding and background staining. The membrane was then washed with TBS-T buffer containing 0.1% Tween and incubated in 20.0 mL of the pool containing IgE serum (1:10) for 5 h under gentle agitation. At the end of the established period, the membrane was washed again with Tween solution to remove any excess non-membrane-bound primary antibody. Secondary antibody (human anti-IgE, specific epsilon chain) conjugated to peroxidase was diluted at 1:200 in TBS buffer. The membrane was incubated in this solution for a period of 4 h under gentle agitation. The presence of effected antibody-antigen bindings was investigated using the Amplified Opti-4CN Kit (Bio-Rad).

Results

C. malaccensis *protein fingerprint*

Ascertaining the protein profile that characterizes a certain mite (fingerprint) is relevant for the development of effective and reproducible immunotherapies in terms of specific protein load. Figure 1 shows the protein footprints of *C. malaccensis*, *D. farinae*, *D. pteronyssinus*, and *B. tropicalis*.

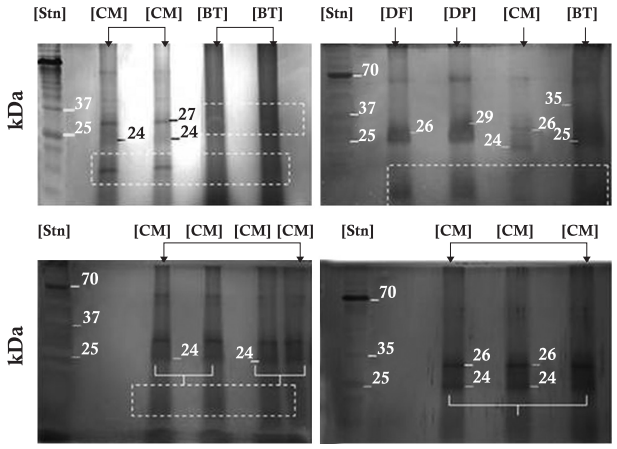

Figure 1
Protein profile of the *Cheyletus malaccensis* mite. (S) standard of Protein-Bio-Rad (Dual Xtra) and PageRuler Broad Range Unstained Protein Laddser (Thermo Scientific), *Dermatophagoides farinae* (DF) (57 µg proteins), *Dermatophagoides pteronyssinus* (DP) (50 µg), and *Blomia tropicalis* (BT) (42 µg)

The protein content of *C. malaccensis* extract was determined by the presence of proteins of molecular weight 24 kDa, 26 kDa, 12 kDa, 45 kDa and 70 kDa. The carbohydrate and protein contents were 0.289 mg·mL-1 and 0.210 mg·mL-1, respectively. It can be inferred that the protein band with molecular mass greater than 70 kDa presented distinct electrophoretic mobility compared to the other bands, possibly characterizing a polysaccharide-linked protein. The 12.5% polyacrylamide gels were applied in extracts obtained on different days by different analysts, confirming the presence of the standard mobility profile and the proteins present in the different extractions. Extracts from *D. farinae*, *D. pteronyssinus* and *B. tropicalis* were applied for comparison in the electrophoretic run. For all three mites, polymorphic group-1 allergens (Df 1, D p 1 and Blo t 1) were found at approximately 25 kDa (acid/neutral protein), classified as cysteine proteinases. Allergens Der f 3, D p 3 and Blo t 3 were identified by molecular weight 29-30 kDa, characterizing trypsin bound to the serine protease enzyme. All of these allergens have been characterized and reported in allergy studies in the scientific literature. Fatty acid-binding proteins were identified by the presence of Der f 2 and Der p 2 allergens of approximately 14-17 kDa (dotted pattern). In *B. tropicalis*, Blot 10, a 35 kDa protein (tropomyosin) was identified.[18,19]

Immunodetection

As shown in Figure 2, after the electrophoretic run, protein transfer to the membrane (a determining step in immunoblotting assays) was confirmed by developing the gel with silver nitrate solution.

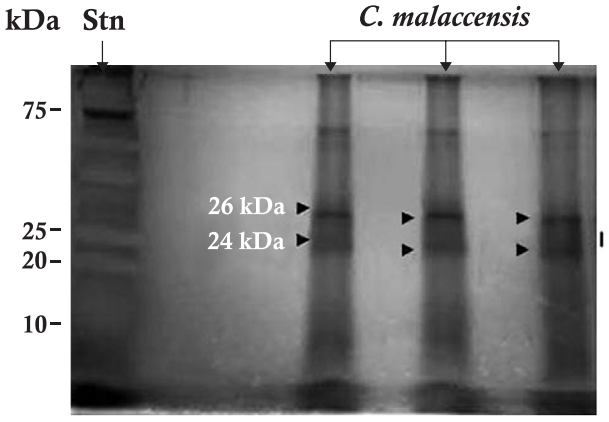

Figure 2
12.5% SDS-PAGE gel. Std, Protein standard (Dual Xtra-Bio-Rad). CM, 0.75 µg of protein extracted from the mite *Cheyletus malaccensis*

Figure 2 depicts a gel similar to that which was transferred to the nitrocellulose membrane and gel after complete transfer of proteins. The reactivity test for serum of individuals sensitized to *D. farinae*, *D. pteronyssinus* and *B. tropicalis* was positive when applied to constituent proteins of the predatory mite *C. malaccensis*, as shown in Figure 3.

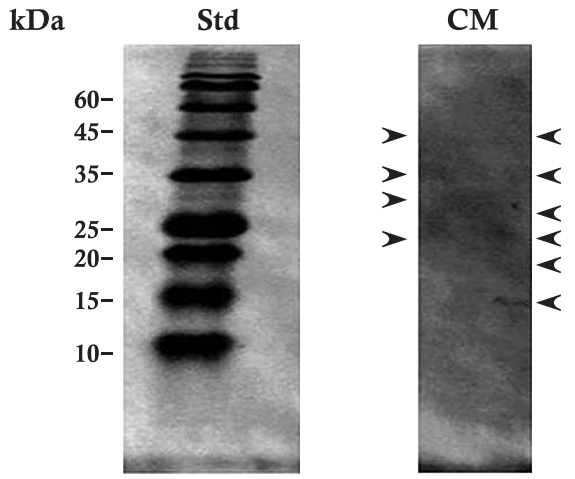

Figure 3
Immunoblotting of *C. malaccensis* proteins with serum of individuals allergic to *D. farinae*, *D. pteronyssinus* and *B. tropicalis*.
Std = standard proteins; CM =*C. malaccensis*

Discussion

The protein bands found in *C. malaccensis* mite extract corresponded to a molecular weight in the range of 20 to 30 kDa (Figure 1), with other proteins at lower concentrations. For all mite extracts, protein bands with a molecular weight <14 kDa, which denotes the presence of peptides, were visualized. The molecular weight range of 20 to 45 kDa, shown in the immunoblot (Figure 3), suggests cross-reactivity between *C. malaccensis* and allergens found in *D. farinae*, *D. pteronyssinus* and *B. tropicalis*. This region contains the cysteine protease group of proteolytic enzymes: Blo t 1 (27.23–25.12 kDa), Der f 1 (25.19 kDa), and Dep p 1 (24.99 kDa), which are present in the fecal extract of the three mites. Many studies have correlated these antigens with respiratory allergies. These studies reported a high degree of potency, in which 87% of positive skin tests for IgE antibodies against cysteine proteases were obtained.[20] The group 3 allergens Blo t 3 (23.82 kDa), Der f 3 (24.95 kDa), and Der p 3 (24.98 kDa) are serine protease enzymes similar to trypsin, with a molecular weight between 23 and 25 kDa. All these allergens have been cited in the literature and are known to trigger allergic reactions.[21-24] It is important to note that, due to taxonomic proximity, there is a degree of homology between the mites under study, corroborating the cross-reactivity demonstrated on immunoblotting. Regarding applicability for immunotherapy, individual use of *C. malaccensis* extract must still be studied further before it can be considered a suitable replacement for pooled *D. farinae*, *D. pteronissinus*, and *Blomia tropicalis* extract. The use of *C. malaccensis* mite extract for routine skin tests, however, is a valid alternative that can be used to determine if an individual is allergic to the other mite species evaluated in this study.

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