

## Millet seed sensitization in a lovebird keeper. A case report.

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Common millet (*Panicum miliaceum* L.) is a highly nutritious cereal used for human consumption, bird seed and/or ethanol production [1], in fact it is the sixth most consumed cereal worldwide: more than a third of the population takes millet from habitual way [2, 3]. Although, nowadays, the grain is known in developed countries mainly as food for birds and livestock.

The anaphylaxis produced by millet was first described in 1981 by Parker *et al.* [4], since then some cases have been described in which this cereal induces serious anaphylactic reactions after ingestion. In addition, millet also could act as an inhalant allergen described for the atopic bird keepers [5, 6]. After the analysis of sera from bird keepers, it was determined that 63% had specific IgE for millet and several bands were detected by immunoblot, mainly three bands that correspond to proteins of 36, 70 and 90 kDa [5, 7]. It was also verified that it presents cross reactivity in case of prolamine, a 20 kDa protein that is common in the different species of millet, as well as in other cereals belonging to the genus *Panicum spp* [8, 9].

We present a case of 35-years-old lovebird keeper with persistent respiratory and nasal symptoms, including ocular lacrimation and severe allergic rhinitis, even causing nasal respiratory failure, as well as dyspnea. Therefore, the specialist's diagnosis was rhinitis and asthma. Previously at the episode described here, the patient was diagnosed with persistent rhinoconjunctivitis due to grass pollen in 2007, and was also treated with SCIT for 5 years with good results. The patient reported not routinely consuming cereals, only some wheat. He also affirmed that the symptoms always got worse at the employ place.

It should be mentioned that the main food of lovebirds was millet, which covered almost 90% of their diet compared with others as birdseed or corn. The withdrawal of millet from the lovebird's diet caused an improvement in the patient's symptoms.

## Methods

### Prick test and specific IgE.

A skin prick test with a standard aeroallergen battery was performed and the study was extended to bird feathers and some cereals that are part of the lovebirds' food.

Subsequently, the specific IgE (Phadia<sup>®</sup> 100 UNICAP Thermo Fisher Scientific) was measured in order to confirm the results obtained with the skin prick test. IgE was measured for millet, corn, birdseed, rye, barley, LTP from peanuts and walnuts and some feathers (canary, parakeet, parrot, etc.).

### Preparation of millet extract.

The allergenic extracts used in ELISA inhibition are routinely manufactured for diagnosis with the exception of millet, which was elaborated following the same manufacturing protocol as with the rest of the extracts. Millet grains of common millet (*P. milliaceum*) (~18g) were crushed and degreased with acetone for 4 hours (25% w/v). After evaporating the acetone, extraction was carried out with phosphate buffered saline (PBS) for 24 hours under stirring and the extract was subsequently filtered by 0.22 µm for sterilization, and subsequent lyophilization.

### SDS-PAGE electrophoresis.

The allergenic extract was analyzed by electrophoresis on SDS-polyacrylamide gels under reducing conditions, according to the eminent technique described by Laemmli in 1970, and its subsequent staining with Coomassie Brilliant Blue R (Sigma-Aldrich<sup>®</sup>).

### Immunoblot.

IgE-immunoblot was performed using corn and millet extract. Briefly, proteins separated by SDS-PAGE were transferred to 0.2 µm nitrocellulose membrane. For immunodetection, membrane was incubated with patient serum diluted 1:5 with blocking solution. And finally, it was incubated with peroxidase enzyme conjugated anti-human IgE (ε chain specific) secondary antibody (Anti-Human IgE-Peroxidase, Sigma-Aldrich<sup>®</sup>).

### ELISA inhibition.

ELISA competition tests were performed in order to assess cross reactivity between allergens, common millet (CAP) (Thermo Fisher Scientific) in solid phase were confronted with a several extracts which act as inhibitors. The ability of millet, corn, wheat, rice and rye extracts to inhibit the binding of specific IgE to common millet was evaluated [10]. The extracts were incubated at 4°C and in continuous shaking with patient serum for 12 hours, and then the inhibition ELISA analyzes were performed (Phadia® 100 UNICAP Thermo Fisher Scientific).

### HPLC-MS/MS

The millet extract and the excised bands of 12 and 36 kDa were analyzed by mass spectrometry coupled to HPLC following an internal protocol according to that described by McCormark *et al.* in 1997. Samples were proteolytically digested with trypsin which decomposed it into peptides which were chromatographed by RP-HPLC (Waters XBridge BEH C18 HPLC column) which was coupled online with an Agilent XCT Plus Ion Trap mass spectrometer using an electro-spray interface. Data processing was performed with a data analysis program for LC/MSD Trap Version 3.3 (Bruker Daltonik, GmbH, Germany) and Spectrum Mill MS Proteomics Workbench (Rev A.03.02.060B, Agilent Technologies).

### Results

#### Skin prick test and specific IgE.

The skin prick tests performed were positive for *Alternaria alternata* and *Cupressaceae*, which could justify the patient's symptoms partially but not totally. For that reason, the study was also extended to feathers and some cereals, being clearly positive for corn, millet and bird seed.

The only significant results for specific IgE were millet and corn, with a value of 1.17 kUA/l and 1.47 kUA/l, respectively. Levels detected for the other tests were very low (<0.39 kUA/l).

### SDS-PAGE electrophoresis.

After separating the proteins present in the sample, the evaluation of the protein profile showed a few bands of different molecular weight. Among them, those of approximate of 12, 20 and 36 kDa were more intense (Figure 1A).

### Immunoblot.

The evaluation of the allergenic profile showed two bands that react with the patient's serum: one of 12 kDa, which has a higher intensity, and another of approximately 36 kDa (Figure 1B).

### ELISA inhibition.

Results showed a very high inhibition of IgE binding to corn, however, the other inhibitions were very low and even null, as in the case of wheat (Figure 1C).

### HPLC-MS/MS

Analysis showed coincidence, with excellent confidence, with non-specific lipid transfer protein from corn (Uniprot B6T089) in the case of band of 12 kDa and uncharacterized protein from millet (Uniprot A0A3L6RCZ2) with homology to glucose and ribitoldehydrogenase in the case of band of 36 kDa.

After all the assays carried out, it can be concluded that the patient is sensitized to 12 kDa protein present in millet and corn, and that it gives rise to cross reactivity demonstrated by ELISA inhibition. The dyspnea reaction suffered may be due to the inhalation of millet seed, since the patient is sensitized to two proteins from the millet extract, approximately 12 and 36 kDa, but this must be confirmed by nasal or bronchial provocation test.

Another case of allergy to millet in a bird keeper is described here.

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## Disclosure statement

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## Figure Legends

Figure 1. A) Protein profile of millet extracts investigated by SDS-PAGE. Extracts were analyzed under reducing conditions, and bands corresponding to main allergens are of 12 and 36 kDa approximately.

B) Immunoblot assay of sera from patient with different dilution and negative control (without extract). The patient presented specific IgE-binding bands with millet. E1: sera diluted 1/5 with blocking solution; E2: sera diluted 1/10 with blocking solution; C- : immunoblot without extract, negative control.

C) Inhibition percentage of millet in solid phase extract (CAP) with extracts of corn, rice, wheat and rye. Millet shows a high inhibition of IgE binding to corn.

