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FIGURE 2: Effect of heat treatment on Der p 2 levels in mite culture

## Study Design

- Aim:** To determine the effects of dry heat on the stability of the major allergen(s) produced by house dust mites, cats and dogs.
- Methods:** Thermal denaturation experiments were carried out by heating allergen source materials, or house dust with high allergen levels, in an oven at temperatures of 60 – 140° C over different time periods (5-60 minutes). Allergens were extracted in saline and the allergen content of the heated sample or a control (unheated) sample, which had been kept at room temperature, was determined by enzyme linked immunosorbent assay (ELISA). The effect of heating the following major allergens was compared: dust mite; Der p 1, Der p 2 and Der f 2; cat, Fel d 1; and dog, Can f 1.
- Results:** Mite allergens: There was a progressive loss of reactivity of the Group 1 allergens with time and increased temperature. Treatment at 100°C for 30 minutes, resulted in a 95% reduction in allergen reactivity in ELISA. Under the same conditions, the Group 2 allergen reactivity was reduced by 53-87%. **Cat and dog allergen:** These allergens were relatively heat stable: Fel d 1 retained 94% of its activity after heating to 120°C; and Can f 1 retained 76% of its reactivity after heating at 100°C.
- Conclusions:** Significant reductions in major allergen content were obtained by dry heating mite culture extract, demonstrating that heat treatments are a substantial adjunct to allergen avoidance regimes. The effects on animal dander allergens were minimal and heat treatment is unlikely to improve cat or dog allergen avoidance procedures.
- Rider:** Therefore a combined thermal (subject paper) and chemical (J ALLERGEN CLIN IMMUNOL 1994; 94:19-26) denaturation procedure will produce significant loss of reactivity of Group 1 and Group 2 mite allergens Der p 1, Der p 2 and Der f 2; cat Fel d 1; and dog Can f 1.

## Methods – Experimental Protocol

Source materials:

*Dermatophagoides pteronyssinus* culture

*Dermatophagoides farinae* culture

House dust extract containing high levels of cat allergen

House dust extract containing high levels of dog allergen

Dog fur clippings

Two aliquots of 100 mg of each allergen source material was placed in heat resistant plastic tubes. One set of tubes was left at room temperature. The test samples were heated for 5, 10, 15, 30 or 60 minutes in an oven at 60°C, 80°C, 100°C, 120°C or 140°C. Samples were extracted in 1ml phosphate buffered saline, pH 7.4, containing 0.05% Tween 20, by rotation for two hours at room temperature. The extracts were centrifuged at 2600rpm for 20min. at 4°C and the supernatant was decanted for allergen assay. The supernatants were stored frozen at -20°C prior to assay.

Allergen assay: Detailed protocols for allergen assay by ELISA follow:-

**ELISA protocol for quantitation of mite  
(Dermatophagoides spp.) Der p 1 or Der f 1**

### **ELISA protocol for quantitation of mite (*Dermatophagoides* spp.) Der p 1 or Der f 1.**

- 1) Resuspend freeze dried mAb 5H8 (anti Der p 1) or 6A8 (anti Der f 1) in 1ml PBS i.e. to 10mg/ml. Coat polystyrene microtiter wells (Immulon II Dynatech) with 1 $\mu$ g/ well of either 5H8 or 6A8 (i.e. 0.1ml 1/1000 dilution of mAb) in 50mM carbonate – bicarbonate buffer, pH9.6, overnight at 4°C.
- 2) Wash wells twice with PBS/0.05% Tween 20, pH 7.4 (PBS-T). Incubate for 1 hour with 0.1ml 1% BSA PBS-T then wash twice with PBS-T and dry.
- 3) Add 0.1ml of diluted allergen samples and incubate for 1 hour. House dust samples are routinely diluted two-fold from 1/10 – 1/80. Use doubling dilutions of a reference *D. pteronyssinus* or *D. farinae* extract to make a control curve. The control curve dilutions should contain from 250 – 0.5ng/ml Der p 1 or Der f 1.
- 4) Wash wells 5x with PBS-T, the incubate for 1 hour with 0.1 ml 1/1000 dilution of biotinylated 4C1 (equivalent to 16ng 4C1 antibody). This monoclonal recognises a common epitope on both allergens and is used as second antibody for both Der p 1 and Der f 1 assays.
- 5) Wash wells 5x and incubate for 30 minutes with 0.1ml 1/1000 dilution of Streptavidin – Peroxidase (Sigma S5512, 0.25mg reconstituted in 1ml distilled water). The Streptavidin should be diluted in 1% BSA PBS-T. Wash wells 5x and develop the assays by adding 0.1ml 1mM ABTS in 70mM citrate phosphate buffer, pH 4.2.
- 6) Stop the reaction after 5 minutes by adding 0.1ml 2mM sodium azide or read the plate when the absorbance (405nm) reaches 2.0 – 2.4. Absorbance readings are directly proportional to the quantity of either Der p 1 or Der f 1 bound and values are interpolated from the respective control curves.

Quantitation of both assays is dependent on the use of mite extracts with known allergen concentration. The Der p 1 standard (UVA 93/03) contains 2500ng/ml Der p 1 and has been sub-standardized against the WHO/IUIS *D. pteronyssinus* reference (NIBSC 82/518), which contains 12.5 $\mu$ g/ml Der p 1. The Der f 1 standard (UVA 93/02) contains 2500ng/ml Der f 1 and was sub-standardized against the WHO/IUIS *D. pteronyssinus* reference using a cross-reacting RIA (as yet, there is no International Reference Preparation for *D. farinae*).

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## Solutions and reagents for ELISA assays

1. 50mM carbonate/ bicarbonate buffer, pH 9.6:

Na <sub>2</sub> CO <sub>3</sub>	1.59g
NaHCO <sub>3</sub>	2.93g

Dissolve in 1 liter deionized water

Thimerosal 0.10g/liter (can be added as preservative if necessary).

2. Phosphate buffered saline, pH7.4, containing 0.05% Tween 20 (PBS-T):

NaCl	8.00g
KH <sub>2</sub> PO <sub>4</sub>	0.20g
Na <sub>2</sub> HPO <sub>4</sub>	1.15g
KCl	0.20g
Thimerosal	0.10g (optional)
Tween 20	0.5ml

All dissolved in 1 liter deionized water. Add 1g bovine serum albumin (BSA, Sigma A-7030) to 100ml PBS-T to make 1% BSA PBS-T.

3. Streptavidin-Peroxidase:

Reconstitute 0.25mg Streptavidin-Peroxidase (Sigma S5512) in 1ml distilled water and store at -20°C in 50µl aliquots. Dilute 1/1000 in 1% BSA PBS-T for use in the assay.

4. Substrate solution, 1mM ABTS in 70mM citrate-phosphate buffer, pH4.2:

70mM citrate-phosphate buffer, pH4.2

Solution A= 0.1M anhydrous citric acid, 19.21g/L

Solution B= 0.2M Dibasic Na Phosphate. 7H<sub>2</sub>O, 53.65 g/L

For 500ml buffer, mix 147ml A + 103ml B and make up to 500ml with deionized H<sub>2</sub>O. Add 274mg ABTS to 500ml buffer to make the substrate solution (contains 1mM ABTS).

ABTS = 2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid), Sigma A1888. The substrate solution is stable at 4°C in the dark. Immediately prior to adding to assay plates, add 1µl 30% H<sub>2</sub>O<sub>2</sub> solution/ ml ABTS. The assay will **not** work if you do not add the H<sub>2</sub>O<sub>2</sub>.

**ELISA protocol for mite  
(Dermatophagoides spp.) Group 2 ALLERGENS**

### **ELISA protocol for mite (*Dermatophagoides* spp.) Group 2 allergens.**

- 1) Coat polystyrene microtiter wells (Immulon II Dynatech, Alexandria, VA, Cat.# 011-010-3450 with 1µg/well of mAb in 50mM carbonate-bicarbonate buffer, pH9.6, overnight at 4°C. The freeze dried mAb should be suspended in 1ml PBS.
- 2) Wash wells 3 times with PBS/0.05% Tween 20, pH 7.4 (PBS-T). Incubate for 1 hour with 0.1ml 1% BSA PBS-T and then wash twice with PBS-T.
- 3) Add 0.1ml of diluted allergen or house dust samples (usually doubling dilution from 1/10-1/80) in 1% BSA PBS-T and incubate for 1 hour. Use doubling dilutions of a reference *D. pteronyssinus* or *D. farinae* extract\* to make control curves. The control curve dilutions should contain from 250 to 0.5ng/ml of Der p 2 or Der f 2.
- 4) Wash wells 5 times with PBS-T, then incubate for 1 hour with 0.1ml biotinylated 7A1 (use 1/3000 dilution for Der p 2 assay and 1/1000 for Der f 2).
- 5) Wash wells 5 times and incubate for 30 minutes with 0.1ml 1/1000 dilution of Streptavidin – Peroxidase (Sigma, St Louis MO, Cat. # S5512; 1mg reconstituted in 4ml distilled water, i.e. at 0.25mg/ml.) Wash wells 5 times and develop the assay by adding 0.1ml 1mM ABTS in 70mM citrate phosphate buffer, pH 4.2, containing 0.03% H<sub>2</sub>O<sub>2</sub>.
- 6) Stop the reaction at 15-20 minutes by adding 0.1 ml 2mM sodium azide or, alternatively, read the plate when the absorbance (405nm) reaches 2.0 – 2.4. Absorbance readings are directly proportional to the quantity of either Der p 2 or Der f 2 bound, and values are interpolated from the respective control curves.

\*Quantification of Group 2 assays is dependent on the use of mite extracts with known Der p 2 or Der f 2 concentration. *D. pteronyssinus* and *D. farinae* extracts can be sub-standardized against CBER/FDA E1-Dp and E1-Df reference preparations, which contain 50µg/ml Der p 2 and 20µg/ml Der f 2, respectively. Although no International References for Group 2 allergens are currently available, we have prepared standards which contain 5µg/ml Der f 2 and can provide these extracts upon request. As the Group 2 assay is cross-reactive, it can be used for detection of both Der p 2 and Der f 2.

## References

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## **RESULTS**

Table 1: Effect of dry heat on house dust mite allergens. The Table shows the results on mite allergen (Group 1 and Group 2) levels obtained after heating *D. pteronyssinus* or *D. farinae* culture at different times and temperatures. The values were obtained by expressing the results as a percentage of the allergen level in control (unheated) samples of mite culture.

Figure 1: Graph showing the effect of heat treatment on Der p 1 levels in mite culture. The dashed line indicates values that are 20% of control values, i.e. a level below which >80% loss of Der p 1 activity has occurred.

Figure 2: Effect of heat treatment on Der p 2 levels in mite culture. In this figure, the dashed line indicates values that are 50% of control, and below which >50% of loss of Der p 2 activity has occurred.