

Dot Immunobinding Assay for Detection of Mite Antigens in House-Dust Samples

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J. Med. Entomol. 35(2): 143-147 (1998)

ABSTRACT A new test was developed specifically to detect mite antigens in house-dust. It uses a nitrocellulose dipstick spotted with specific antimite antibodies that act as a capture matrix; the same antibodies act as a detecting reagent when conjugated with colloidal dye particles. Aclo test is a 1-step assay, where a spotted dipstick is placed in a tube containing the detecting reagent and the house-dust sample. No instrumentation or previous extraction procedure of the sample is required, and the test response is visible as a colored spot, after 1 h incubation. The sensitivity and specificity of the new test were compared with those of Acarex and Der p1/Der f1 ELISA tests.

KEY WORDS mite antigens, dot immunobinding, Aclo test, colloidal-dye, dipstick

ALLERGY TO HOUSE dust mites, in particular *Dermatophagoides pteronyssinus* (Trouessart) and *D. farinae* Hughes, is a worldwide problem (Platts-Mills 1987). Colonies of house-dust mites probably live in every home, but some environmental conditions such as warmth and moisture, upholstered furniture, carpets, mattresses, bedding, curtains, and soft toys offer the best environment for their survival. Because the magnitude of exposure to mites is important for sensitization and the development of symptoms (Korsgaard 1983, Platt-Mills et al. 1985), simple, specific, and rapid methods to assess their presence in the domestic environment would help in selecting the best antimite measures to minimize allergen exposure and limit allergic symptoms and the need for medication.

Current methods for this purpose are not completely satisfactory. For example, microscopic observation is time-consuming and needs highly skilled personnel. The recently developed Acarex test (Kallergen, Settino Milanese, Milan, Italy) (Bronswijk 1986) is rapid and simple, and patients can administer it themselves, but it is limited because of its low specificity (Vervloet et al. 1989, Mistrello et al. 1992). The immunoenzymatic assay described by Luczynska et al. (1989) is the most sensitive and specific assay, but unfortunately the sample first has to be extracted and therefore requires specific instrumentation.

Here we describe a new, rapid, and simple test (Aclo test) that specifically detects mite antigens in dust samples.

Materials and Methods

Preparation of *D. pteronyssinus* or *D. farinae* Extracts and of Specific Anti-*D. pteronyssinus* Rabbit

Serum. Whole bodies of *D. pteronyssinus* or *D. farinae* mite cultures were separated from the culture medium by passages through serial sieves at different mesh sizes. Under these conditions, mite bodies amounted to >95% of purity, as established by microscopic observation. *D. pteronyssinus* and *D. farinae* material was homogenized in phosphate buffered saline (PBS) (10% wt:vol) pH 7.4, with 0.05% NaNa₃; suspensions were stirred at 4°C overnight and centrifuged at 20,000 × *g* for 30 min at 4°C. Supernatants were then gel-filtered on Sephadex G-25 columns and the peaks were collected and used as mite extracts. The protein content of *D. pteronyssinus* or *D. farinae* extracts was determined according to Bradford (1976) and the Der p1 content of *D. pteronyssinus* extract was quantitated according to Luczynska et al. (1989). To obtain specific anti-*D. pteronyssinus* rabbit serum, 3 mg of *D. pteronyssinus* extract with either complete (first 2 immunizations) or incomplete (boosters) Freund's adjuvant was injected subcutaneously into rabbits 4 times at monthly intervals. One week after the last dose, blood was collected and pooled. Serum IgG was precipitated by ammonium sulphate, and anti-mite specific antibodies were purified by affinity chromatography as previously described (Mistrello et al. 1993).

Purification of Der p1/p2 Allergens. Der p1 or Der p2 allergens were obtained by affinity chromatography. Immunoabsorbents were prepared by coupling separately the 50% saturated ammonium sulphate precipitate of anti-Der p1 or p2 specific Mab (4C1 and 7A1) to CNBr activated Sepharose (Pharmacia, Cologno Monzese, Milan, Italy). *D. pteronyssinus* mite extract was applied over the column and Der p1 or p2 allergen, from the respective immunoabsorbents, was eluted with 0.005 M glycine

in 50% ethylene-glycol, pH 10. Pooled fractions were dialyzed against 5 mM ammonium bicarbonate and lyophilized.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting. Electrophoresis was done according to Laemmli (1970), by using a linear 12% polyacrylamide gel in a Mini gel SW 250 Hoefer apparatus. The samples (MW standards, *D. pteronyssinus* and *D. farinae* extracts and a mixed solution of Der p1/p2 affinity purified allergens) were diluted in running buffer (Tris-HCl 0.125 M, pH 6.8, 5% β -mercaptoethanol, and 2% SDS) and then boiled for 3 min. The resolved proteic components were blotted according to Towbin et al. (1979) onto Fluorotrans membrane (0.45 μ m, Pall, Milan) using a WB2 electroblotting apparatus (Prodotti Gianni, Milan, Italy) at 40 V for 3 h. After 2 h of saturation with Tris 20 mM, NaCl 0.9% containing 5% fat-free milk, the transferred proteins were incubated with specific rabbit antimite antibodies diluted at 1:1000. Bound IgG were revealed by adding anti-rabbit IgG peroxidase (1:1000) using 3'-3'-diaminobenzidinetetrahydrochloride (DAB) as chromogen and H_2O_2 as substrate. Both antibodies were diluted in saturation buffer.

Preparation of Dispersed Dye. A dye particle suspension (Samaron Brilliant Pink, Hoechst, Milan) was prepared as previously described (Mistrello et al. 1995). Briefly, 5 g of textile dye was suspended in 100 ml of distilled water and centrifuged; the pellet was washed 3 times and then centrifuged at low speed ($1,500 \times g$) to remove aggregated colloidal particles. To establish a reference parameter (A), a sample of colloidal dye particles was dissolved in ethanol and measured at 540 nm. The dilution of colloidal dye necessary to obtain an optical density of 1 was indicated as A.

Preparation of the Colloidal-Dye/Antibody (Abs) Conjugate. Different concentrations of specific antimite antibodies (in μ g/ml) and different dilutions of colloidal dye particles (A value) were mixed in a final buffer 10 mM phosphate, 10 mM NaCl, pH 7.4, to define the optimal ratio of the reagents. After 1 h incubation at room temperature to allow adsorption of antimite antibodies onto colloidal dye particles, a solution of 5 mM NaCl pH 7.4 + 30% BSA, corresponding to 1/5 of the initial volume, was added to the dye-Abs suspension to stabilize the colloidal form; the suspension was centrifuged at $12,500 \times g$ for 20 min at 4°C. The dye/Abs pellet was resuspended in 33.3 mM phosphate, 0.125 M NaCl solution, pH 7.4, containing 5% BSA and 0.01% thiomersal, and lyophilized before use.

Preparation of Anti-*D. pteronyssinus* Antibody Coated-Sticks. Five micrograms of specific antibodies in 8 μ l of PBS was spotted onto nitrocellulose reactive areas (0.8 by 0.8 cm) stuck on plastic sticks. The sticks were dried and saturated for 2 h with Blocker Blotto (Pierce, Prodotti Gianni, Milan, Italy), then washed with distilled water, dried, and stored at 4°C.

AcloTest Assay. Preliminary experiments were done to establish the best conditions for using the dye/Abs conjugate. An artificially positive house-dust sample was prepared by mixing 1 part of *D. pteronyssinus* mites as whole culture with 2 parts of house-dust, negative in Der p1/fl ELISA assays and by microscopic observation.

The AcloTest procedure provides 1 ml of dye/Abs conjugate at different dilutions/concentrations, which was placed in tubes containing ≈ 80 mg of the positive house-dust sample. Anti-*D. pteronyssinus* specific antibody-coated sticks were immediately put into these tubes, incubated for 1 h, then washed with tap water, after which a pink spot appeared on the dipstick. The dye/Abs conjugate combination giving the maximum color intensity with the minimum background was chosen and used to check the sensitivity of the AcloTest.

Sensitivity of AcloTest. To define the sensitivity of the AcloTest test, 1 ml of dye/Abs conjugate was added to tubes containing lyophilized *D. pteronyssinus* extract samples at various concentrations of Der p1. Taking into account the different intensities of the color of the spots on the dipstick, an arbitrary positivity scale was obtained. This scale was then used to establish the values of house-dust samples to be checked.

Preparation of House-Dust Samples. House-dust samples were collected after shaking up the mattress in the bedroom of the individuals involved in this study. House-dust corpuscular material (e.g., dust without fluff) was weighed, divided into 3 equal parts, and tested, using Acarex, AcloTest, and Der p1/fl ELISA, each on one part.

Acarex Test Procedure. The test was carried out according to the manufacturer's instructions (Kalergen).

ELISA for Der p1/fl. The assay was performed following the procedure of Luczynska et al. (1989). Briefly, microtiter plates coated with specific MAb (5H8 or 6A8) were saturated after washings with 100 μ l of 1% PBS-BSA. Subsequently, 100 μ l of $2 \times$ serial dilutions from 250 to 1.5 ng/ml of reference *D. pteronyssinus*/*D. farinae* samples or *D. pteronyssinus*/*D. farinae* mite extracts or house-dust samples were added. After further washings, biotinylated specific Mab was added followed by streptavidin-peroxidase solution. Bound biotinylated-labeled antibody was detected using 2',2'-azino-bis(3-ethylbenzthiazoline)sulfonic acid as chromogen and H_2O_2 as substrate. Optical density was read after 5 min using a microplate spectrophotometer at 450 nm, and Der p1/fl contents were determined by interpolation from the reference curve.

Results

Specificity of Anti-*D. pteronyssinus* Rabbit Serum. Anti-*D. pteronyssinus* rabbit serum was tested by immunoblotting experiments against mite extracts (*D. pteronyssinus* and *D. farinae*) to evaluate the components recognized by specific antibodies.

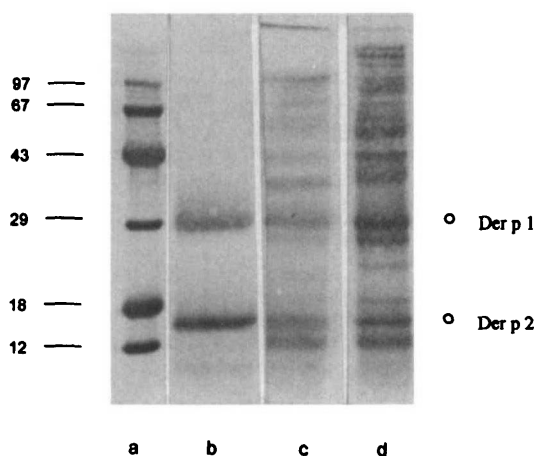


Fig. 1. Specific anti-*D. pteronyssinus* serum was tested in Western blot experiments using Der p1/p2 mixed affinity purified allergens (lane b), *D. pteronyssinus* extract (lane c) and *D. farinae* extract (lane d). a, m.w. standard; b, Der p1/Der p2 purified allergens; c, Df extract; d, Dp extract.

Several bands in a wide range of molecular weights were visible (Fig. 1, lane c). Der p1 and p2 allergens were particularly well recognized. Their position in the *D. pteronyssinus* extract immunoblotting profile was established by comparing it with one obtained using a mixture of Der p1/p2 affinity purified allergens (14 and 29 kDa). When anti-*D. pteronyssinus* serum was tested against *D. farinae* extract, the pattern was very similar to that of *D. pteronyssinus*, indicating clear cross-reactivity between *D. pteronyssinus* and *D. farinae* mite antigens.

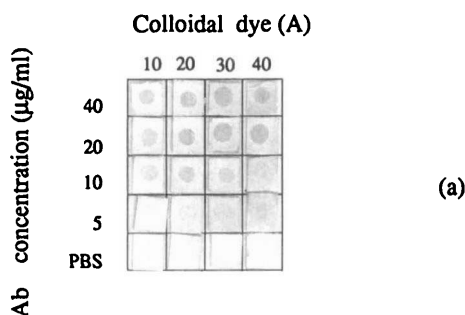
Acloctest Optimization. Different textile dyes, such as Samaron Red, Palanil Dark Blue, and Samaron Brilliant Pink, were tested to select the best. Optimal conjugation between textile dye and antibody was obtained using Samaron Brilliant Pink. In preliminary experiments we established the optimal ratio of conjugation between colloidal dye (A) and specific polyclonal antibodies. As shown in Fig. 2a, a wide range of dilutions of dye particles (from A = 10 to A = 40) were tested, with different concentrations of antibodies (from Ab = 5 μ g/ml to Ab = 40 μ g/ml). The most satisfactory result, giving the maximum response as a colored spot with the minimum background, was obtained with A = 30 for the colloidal dye and 20 μ g/ml as antibody concentration.

In the negative control, PBS was substituted for the specific antibody in the conjugation procedure. In this case, no colored spot appears, confirming that colloidal dye alone does not induce reaction.

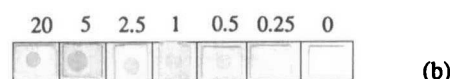
These data were obtained using nitrocellulose sticks spotted with a fixed concentration of antibody (5 μ g/8 μ l) known to be optimal in other dot-blot experiments.

To define the sensitivity of Acloctest, *D. pteronyssinus* extract samples with a titrated Der p1 content

Optimization Dye/Abs Conjugate



Sensitivity (μ g/g Der p1) ACLOTTEST



Arbitrary Positivity Scale (μ g/g Der p1)

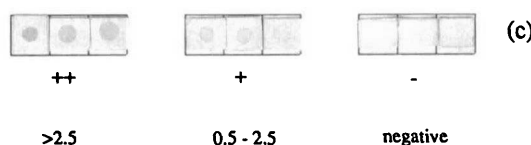


Fig. 2. (a) Optimization in the preparation of colloidal dye-Ab (specific anti-*D. pteronyssinus* antibodies) conjugated. (b) Sensitivity of Acloctest (μ g/g as Der p1). (c) Arbitrary positivity scale of Acloctest.

(concentrations varies from 0.25 to 20 μ g/g in terms of Der p1) were tested. Acloctest was able to detect up to 0.5 μ g/g of Der p1 (Fig. 2b). On the basis of these results and taking into account the different intensity of the color, the following arbitrary positivity scale can be defined: ++ for >2.5 μ g/g Der p1; + for between 0.5 and 2.5; and - for negative (Fig. 2c).

Comparison of Acloctest, Acarex, and ELISA (Der p1/Der f1). Table 1 sets out the results of analysis of 50 house-dust samples. The Acloctest found 10 out of 50 samples were negative, and positive values were + or ++. The Acarex test found mites in all samples, with different degrees of positivity from low (39 samples) to high (11 samples).

To confirm the real positivity or negativity of the samples tested, we used the Der p1/f1 ELISA method, which is acknowledged as the most reliable in view of its sensitivity and specificity. Five of the samples negative by Acloctest (numbers 3, 9, 22, 29, and 34) were confirmed as negative; the others (numbers 21, 27, 28, 35, and 37) were positive as Der p1 ELISA, through with Der p1 levels near the Acloctest sensitivity threshold, 0.5 μ g/g.

Table 1. Comparison among ELISA Der p1/f1, Acclotest, and Acarex

Samples	Acclotest	Acarex	Derp 1/Derf 1	($\mu\text{g/g}$)
1	+	R	2	—
2	++	R	5	—
3	Negative	R	Negative	Negative
4	++	R	4	—
5	++	H/R	>20	—
6	++	R	5	—
7	+	R	2	—
8	++	R	8	—
9	Negative	R	Negative	Negative
10	++	R	5	—
11	++	H/R	5	—
12	++	R	3.5	—
13	++	H/R	>20	—
14	++	R	5	—
15	++	R	>20	—
16	+	R	3	—
17	++	R	5	—
18	++	R	2.5	—
19	+	H/R	2	—
20	+	H	2	—
21	Negative	R	0.2	—
22	Negative	R	Negative	Negative
23	+	H/R	2.5	—
24	+	R	Negative	1.5
25	++	R	6	—
26	+	R	1	—
27	Negative	R	0.5	—
28	+	R	0.5	—
29	Negative	R	Negative	Negative
30	+	R	Negative	2.0
31	++	R	6	—
32	+	R	0.8	—
33	++	R	3.5	—
34	Negative	R	Negative	Negative
35	Negative	R	0.2	—
36	++	H/R	>20	—
37	Negative	R	0.2	—
38	+	R	1.4	—
39	++	H	>20	—
40	++	R	2.8	—
41	++	R	10	—
42	++	R	7.4	—
43	++	H	>20	—
44	+	R	0.8	—
45	++	H	>20	—
46	+	R	1	—
47	+	R	1.5	—
48	++	H/R	>20	—
49	+	R	0.9	—
50	+	R	2.3	—

++, >2.5 $\mu\text{g/g}$ Der p1; +, <2.5 >0.5; negative, <0.5; —, not tested; R, reduced; H, high.

An apparent contradiction between Acclotest and Der p1 ELISA, regarding 2 samples (numbers 24 and 30), was overcome by using Der f1 ELISA, which established that they were positive.

Discussion

The role of house-dust mites and their fecal particles as a cause of human allergic pathology has been unequivocally demonstrated (Platts-Mills et al. 1987, Tovey et al. 1981). Modern dwellings are ideal habitats for survival and growth of these mites. Prolonged exposure to certain levels of indoor mite allergens increases the risk of allergic sensitization

in normal populations and of symptoms in allergic people (Platts-Mills et al. 1985, Korsgaard 1983).

Avoidance of the allergen is the best line of defense against allergy. Avoidance of indoor mite allergens is relatively easy if patients use all the preventive measures now available—for example, acaridic products. The indoor distribution of house-dust mites tends to vary so measures aimed at locating the places infested with mites can be useful to target cleaning operations.

Apart from microscopic observation, which is not suitable for routine analysis, a rapid and simple test has been developed recently to be administered by patients themselves (Bronswijk 1986). This assay, the Acarex test, is however not optimal in terms of specificity (Vervloet et al. 1989, Mistrello et al. 1992), probably because it relies on the determination of guanine, the end-product of purine catabolism of mites but in general of all arachnids including spiders. This lack of specificity is confirmed by our experiments where some house-dust samples were negative both to Der p1/f1 ELISA and Acclotest, but were positive in the Acarex test.

In view of its sensitivity and specificity, we thought it is reasonable to consider Der p1/f1 ELISA as the reference assay to establish the negativity or positivity of a dust sample. On this basis, our data suggest that a good degree of correlation between immunoenzymatic assay and Acclotest exists, whereas Acarex gave false-positive results in some cases.

The Acclotest is a new, specific assay that combines 2 different technologies, dot-blot and colloidal dye-conjugated protein, for direct visualization of the response (Gribnau et al. 1991, Mistrello et al. 1995). This test is rapid and easily executed. Acclotest is less sensitive than the Der p1/f1 assay, but in any case it detects mite allergen at levels known to be associated with a risk of allergic sensitization (2 μg Der p1/g dust). However, the Acclotest offers some advantages over the immunoenzymatic assay. Starting directly from the house-dust samples, it needs no extraction procedure or any of the instrumentation required for immunoenzymatic assay.

An interesting finding came from 2 samples (Table 1, numbers 24 and 30) in our study. There seemed to be a contradiction between the Acclotest (positive) and Der p1 ELISA (negative) finding, but analysis by Der f1 ELISA confirmed they were both positive in accordance with the Acclotest results. This should not be a surprise. Our immunoblotting experiments (Fig. 1) using anti-*D. pteronyssinus* serum clearly indicated close cross-reactivity between *D. pteronyssinus* and *D. farinae* antigens and a similar antigenic structure between group 1 (Der p1/f1) and 2 (Der p2/f2) is known (Heyman et al. 1989, Robinson et al. 1997). This does not exclude that Der p1 and Der f1 allergens can have original epitopes recognizable by specific monoclonal antibodies such as the ones used in the Der p1/f1 ELISA assay.

This observation implies another possible advantage of Aclorest, i.e., that a single assay may be enough to detect Der p1/fl allergens, which on the contrary require 2 specific assays by the ELISA method.

Based on these results we conclude that Aclorest is useful and simple for detecting house mites. This information could be very useful to help clinicians plan the most effective measures for avoiding allergic sensitization or eruptions of symptoms.

Acknowledgments

We are grateful to Giuseppe Centanni for help and criticisms, Martin D. Chapman (University of Virginia, Charlottesville) for providing anti-Der p1/fl monoclonal antibodies, Paola Lodigiani for typing the manuscript, and Judy Baggott for revision of the English text.

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Received for publication 5 November 1996; accepted 3 October 1997.