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Direct Allergy Test (DAT) – New method for detection of antigen-antibody reactivity

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Abstract: *An alternative for the tests of immunodetection diagnoses is proposed in this work, based on the elaboration of a qualitative method that can aid in the identification of antigen-antibody immunochemical reactivity in rapid diagnoses of allergies. For this purpose, the allergen-containing Dermatophagoides farinae mite extract was immobilized with glutaraldehyde on reusable support and blocking steps, addition of antibody containing serum to the allergens present in the mite extract. The reactivity response was obtained after colorimetric revelation. The appearance of the purple color signaled the reactivity between antigen-antibody on the plate. The colored area was photographed and the reactivity was represented by the intensity of color in pixels using the gel analyzer software. The method was evaluated by repeatability tests, negative assay and variation in protein content. From the proposed method a positive reactivity response was verified for the serum of individuals sensitized by mite. The method showed better performance for application of the protein load of 500 µg of the mite extract, 0.50% (v/v) glutaraldehyde and 0.25% (w/v) blocking. Repeated analyzes performed on different days showed that the method is reproducible being in agreement when compared to the immunoblotting technique. The results obtained for the development of the antigen-antibody detection method showed its promising potential as an alternative aid in the screening of antigen-antibody allergic reactions. The qualitative method, however, does not aim at replacing traditional techniques, but it provides speed and cost reduction, as it eliminates the electrophoretic separation step, dispenses standard purified proteins, and reuses the support, besides providing less reagent consumption.*

Keywords: *immunodetection, diagnostics, antigen-antibody, allergies.*

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I. Introduction

Western Blotting is based on the recognition of specific biomolecules, such as antigens and antibodies (immunoglobulins), including the appearance of autoimmune, infectious and allergic diseases[1,2]. Among the immunoblotting application areas, there are diagnostic tests for allergies triggered by mites (asthma, sinusitis, bronchitis) [3-5]. The classical allergy diagnostic methods, such as WB and Enzyme – Linked Immunosorbent Assay (ELISA), aim to identify the antigen – body reactivity with sensitivity and specificity [6-8]. However, these techniques require a long time of execution, requiring for WB the separation of proteins by electrophoresis and subsequent transfer to membranes (nitrocellulose or polyvinylidene fluoride)[9,10]. In contrast, the ELISA technique requires standard curves for the allergens (purified antigens), in which it is desired to determine, thus making the analysis process more expensive. Through all these problems the present study proposes an alternative method for the detection of allergenic proteins, called Direct Allergy Test (DAT). The technique is based on the elaboration of a reusable silica (glass) base, which does not require the use of membranes, and there is no need for standard marker proteins. The method detects the presence of allergenic proteins in the serum of atopic individuals qualitatively. In the case of the recognition mechanism, reactions between antigen-antibody (epitope and specific immunoglobulin) occur through relatively weak bonds (Van der Waals force) and hydrogen bonds, being boosted by the increase in the number of interconnections, which is the basic principle for the proposed technique. The DAT method is a simple and quick alternative, with reduced antigen sensitized individuals diagnostics costs. The present study uses the Dermatophagoides farinae (Df) mite extract as a source of allergens, being cited in literature as one of the main causes of atopy (asthma and renitus) in tropical countries (Africa, West Indies, Asia, South America and Latin America) [11,12].

II. Material And Methods

Mite Cultivation

Dust samples were collected in households located in the city of Rio de Janeiro, Brazil. The particulate material, obtained in the mechanical aspiration (portable aspirator, NV3600, Black & Decker) was sieved in 80 mesh. In these samples, the taxonomy was identified and classified by optical microscopy for the Dermatophagoides farinae (Df) mite[13]. From the identification, the artificial cultivation of the species was started using a container with fish feed for a period of 90 to 180 days at room temperature (25 ° C). After this time, the mass of this mite was separated from the residues by a 40 mesh sieve, using ether as a sterilizing agent and subsequent dehydration.

Extraction and protein content determination

The protein extract was prepared from 10% (w / v) of the mite's mass in saline buffer solution (TBS) at pH 7.0. For structural disruption a sonicator was used (ultrasound for 30 minutes at the frequency of 20 kHz), followed by a pH adjustment to 8.5. The mixture then stood for 48 hours under refrigeration. After that time, it was centrifuged at 1500 xg for 30 minutes and the supernatant collected. The supernatant was filtered with 0.22 µm cellulose acetate membrane (Millipore, Brazil), and the pH adjusted to 7.0. The protein content was determined by the Lowry method as described by USP-32[14], using bovine albumin as standard. The total protein content was calculated using the linear regression method from the analytical curve. The extract was concentrated by precipitation with acetone and the protein content adjusted, when necessary, by dilution.

Use of *Trichophyton rubrum* as negative control

In order to guarantee the reliability of the results, a challenge was proposed using an allergenic extract from another source, the fungus Trichophyton rubrum (T. *rubrum*) as negative test, following the same procedure of immobilization for the D.fariane mite extract. For the extract of T. rubrum the absence of color in the development stage is expected, which confirms the non-reactivity. For this experiment, an extract with a protein content of 23.0 mg mL^{-1} was used, and diluted when necessary.

Characterization of protein extracts by electrophoresis in denaturing conditions

The *Dermatofagoides farinae* extract protein fingerprint was determined by electrophoresis under denaturing conditions using sodium dodecylsulfate and mercaptoethanol, according to literature [15]. The same procedure was used to characterize the extract of the fungus *Trichophyton rubrum* yielded by the Laboratory of Extract Allergens Ltda - Brazil. The gel was prepared from the polymerization of the acrylamide reagent to polyacrylamide according to the following protocol: for the 0.5 mL gel 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), already for the gel where the separation of proteins occurs (lower gel) the percentage of acrylamide added was performed by addition of 4.125 mL Acrylamide / BiS-Acrylamide 30% solution, (29: 1); 5.0 mL 0.75 M Tris-HCl-SDS Buffer / pH 8.8; 75 µl APS (Ammonium persulfate): 10 % (w / v); 50 µL TEMED and 0.8 mL Distilled water. For the application of the sample, 30.0 µL of the sample of *D. farinae* extracts were removed and 15.0 µL of reducing buffer containing 0.10 M Tris-Cl, pH 8.45, 1.0% (w / v) SDS, 2 % (v / v) 2-mercaptoethanol, 20% (w / v) glycerol, 0.04% and bromophenol blue. For comparison of the migrations a standard color protein marker (Dual Xtra-Bio- Rad) was used with molecular weight ranging from 250 to 10 kDa, which was applied in the gelAnalizer10a software to determine the molecular mass (kDa) of the extracts' constituent proteins.

Immobilization of the antigen and addition of the antibody

On the silica (glass) support delimited by an area of 6.0 cm^2, 100 µL of the glutaraldehyde mixture (0.50% (v / v)) / BSA 1: 1 (v / v) was homogeneously applied. Then 100 µL of the 5.03 mg mL^{-1} protein extract was immobilized onto the surface area of the support and allowed to dry for 1 h. To avoid non-specific binding by the primary antibody (serum) the bovine serum albumin standard of 0.5 mg mL was used as a blocking in the immobilization step.

The serum pool of 15 individuals, allergic to the D. fariane mite was diluted 1:20 in 0.10 mol L-1 saline buffer and a volume of 100 µL was applied as the antibody source for reactivity in the proposed system. Excess serum (unbound antibody) was removed by washing with TBS-T buffer pH 7.5.

Immuno Assay Revealing

Effective binding between antigen and specific IgE was confirmed by the application of 18.7 mg mL^{-1} anti-human serum (Sigma-Aldrich, Australia) containing the conjugated peroxidase enzyme. The anti-human serum was diluted 1: 100 (v / v) in TBS buffer pH 7.5 and the substrate developing solution was prepared from 4-chloro-1-naphthol, 33.59 $mmolL^{-1}$ (Sigma- Aldrich, Brazil) and 0.20 % (v / v) H_2O_2 (Sigma-Aldrich, Brazil).

The tests were considered positive and the antigen-antibody reactivity effected by the appearance of the purple color.

Repeatability test

To evaluate the repeatability of the DAT method, five experiments were carried out on the same day with reagent solutions prepared at the time of the analysis[16]. To confirm the absence of false positive reactions, the protein extract of the Trichophyton rubrum fungus, not reactive to the specific immunoglobulin present in the serum of sensitive individuals *to D. farinae* mite was used. What is expected is that the proteins (epitopes) present in this extract do not bind to IgE specific allergens and therefore do not produce a positive result (purple coloration). However, if the blockade is not efficient, the false positive result will occur, confirming nonspecific IgE binding on the glass plate. Thus, the blocking step with BSA (inert protein) is considered critical in order to obtain an efficient result in DAT.

Comparison between Western blot methods and Direct Allergy Test

In order to compare and validate the assays, immunodetection tests were performed using Western blot and the proposed DAT methodology. Considering that there is reactivity between *D. farinae* mite allergens and specific IgE, the appearance of purple staining was considered an indicator of positive reactivity for both techniques. In all analyzes the negative controls were added.

Apparatus and procedure for the application of the Direct Allergy Test

Immobilization support for the development of the Direct Allergy Test (DAT) method was prepared from a silica (glass) base with an acrylic side apparatus with an area of 1.53 cm^2 delimited for the application of the reagents as shown in **Figure 1**. The sequence of procedures follows in order:

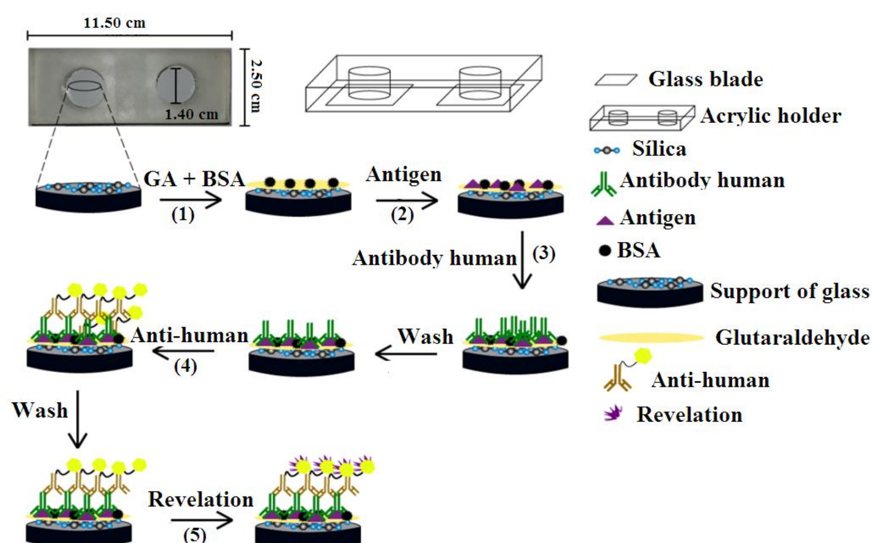
1. Addition of glutaraldehyde (GA) together with bovine serum albumin (BSA) and subsequent addition of 100 µL of antigen-containing *D. farinae* extract (5.0 mg mL^{-1} total protein);

2. After drying (1 h) 100 µl of serum containing specific immunoglobulin (IgE) diluted 1:20 were added;

3. After washing with saline buffer containing Tween (1% (v / v)), 100 µL (1: 100) of anti-human (anti-IgE) enzyme-conjugated peroxidase;

4. Finally, 200 µl of 4-chloro-1-naphthol 0.60 mg mL^{-1} (revealing solution) was added;

5. To perform the test (negative) 100 µL of extract of the Trichophyton rubrum fungus with protein content of 2.3 $mgmL^{-1}$. Each procedure was repeated in three steps as described above.



Figurenº 1: Procedure steps for antigen immobilization, blocking and antibody addition. (1-2) Addition of the immobilizing agent (glutaraldehyde - GA) with bovine serum albumin followed by subsequent addition of the allergenic extract. (3) Addition of serum containing IgE (specific immunoglobulin). (4) Addition of conjugated anti-human to peroxidase enzyme. (5) Colorimetric revealing (purple color).

III. Result

The allergens present in the body and feces of the *D. farinae* mite are already well known and determined by a large number of research studies in the field of allergy and immunology, from which the electrophoretic bands and their respective molecular mass (kDa) were characterized, as in **Figure 2A**.

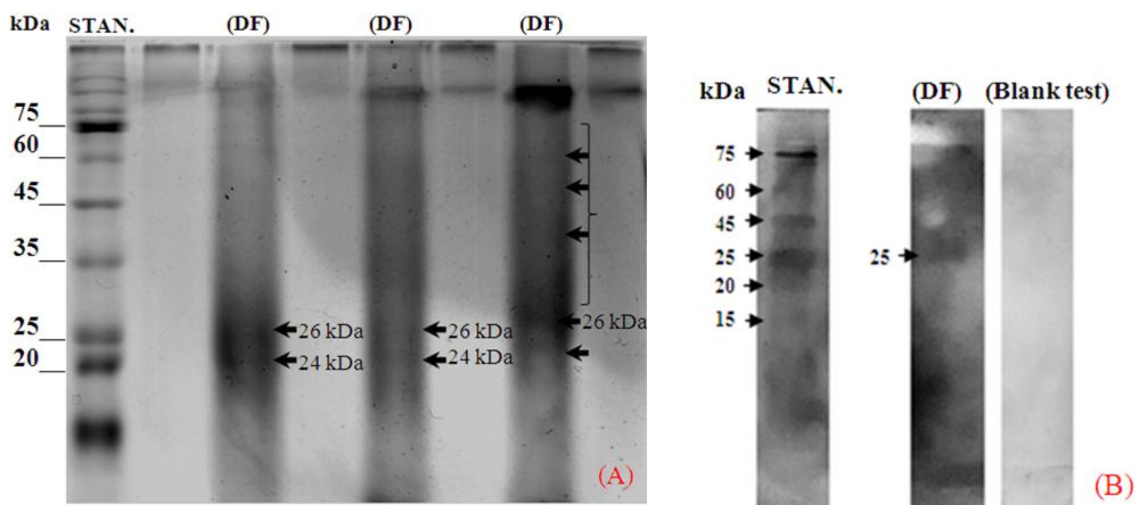


Figure nº 2: (A) Electrophoretic separation by the SDS-PAGE method 12.5%. STAN - Pattern of standard marker proteins (Truecolor high protein protein marker), DF - proteins present in the extract of the *Dermatofagoides farinae* mite (250.0 μg protein). (B) Western Blotting. 0.45 μm nitrocellulose membrane containing proteins (allergens) in the presence of *Dermatofagoide farinae* (Df) and *Trichophyton rubrum* (Thy) fungus STAN: True Marker Protein Marker (Df): positive reactivity for specific immunoglobulin (IgE) and (Thy) negative reactivity for IgE present in the serum of the sensitized individual by DF (bench test).

Repeatability test for the DAT method

The results obtained for the repeatability test were performed in quintuplicates (n = 5) as shown in **Figure 3** for antigen immunodetection (*Dermatophagoides farinae* mite extract) and antibody (specific immunoglobulin present in the serum of a diagnosed mite allergic subject).

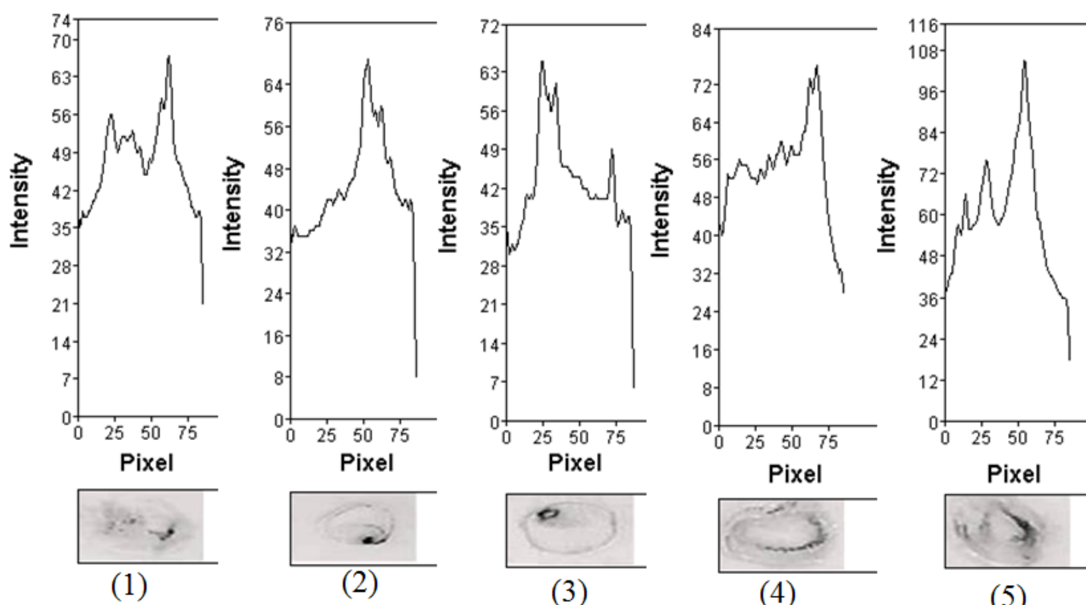
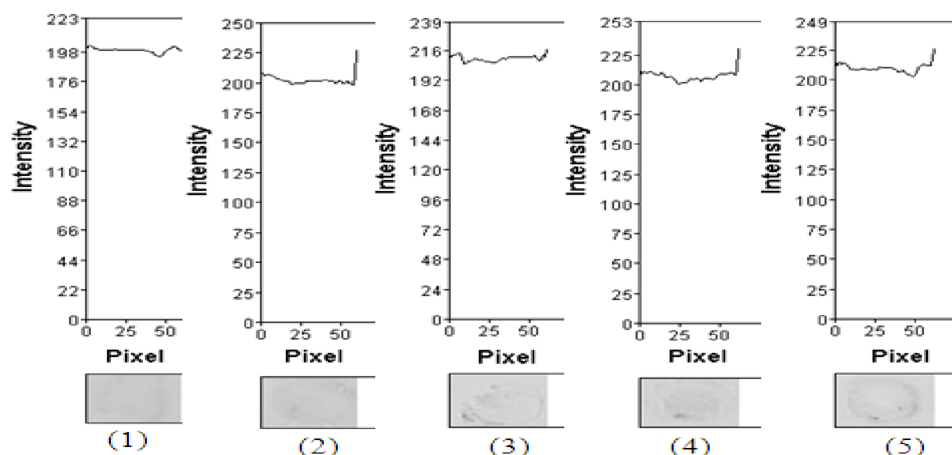


Figure nº 3: Reactivity positive to the antibody-DAT method. Repeatability (n = 5) for the DAT method using serum containing immunoglobulin specific for *Dermatophagoides farinae* (0.50 mg of total protein).



Figuren°4: Negative reactivity for the antigen negative test (proteins present in the extract in contact with IgE present in the serum of individuals sensitized to the *D. fariane* mite. The blank test (negative) was carried out applying as a protein source the extract of the fungus *Trichophyton rubrum* with 2.3 mg mL^{-1} concentration.

Influence of the immobilized protein content for *D. farinae*

The staining intensity versus pixel data are shown in **Figure 5**. This graph represents the reactivity response for individual serum with IgE specific for *D. farinae* in the presence of the following protein contents: 0.5 mg; 1.0 mg; 1.5 mg; 2.0 mg and 2.5 mg.

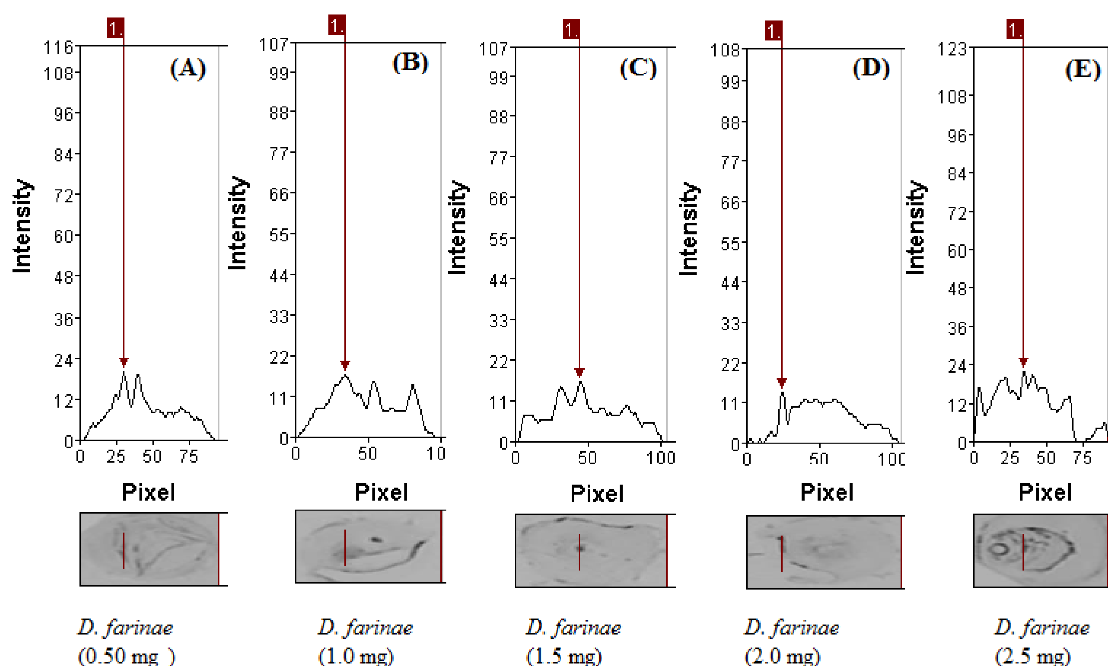


Figure nº 5: Reactivity obtained by the Direct Allergy Test (DAT) method by subtraction of the baseline represented by the blank test. Application of IgE present in serum (1:20) of *D. farinae*-sensitized individual in the presence of antigens (present in *D. farinae* mite extract) immobilized on support at the following concentrations: (A) 0.500 mg; (B) 1.00 mg; (C) 1.50 mg; (D) 2.00 mg and (E) 2.50 mg with blockade of 0.25% (w / v) BSA and glutaraldehyde 0.50% (v / v).

IV. Discussion

As shown in **Figure 2A**, the presence of the allergen 25, kDa, of group 1, Der f 1, was identified in the protein extract of the *D. farinae* mite, characterized by belonging to the cysteine protease enzyme group and is known to be a trigger of allergies in at least 70% of mite-sensitized individuals[13], being present in the body and feces of these microorganisms. The extract also shows group 4 allergens of the glycosidases having alpha-

amylase, Der f 4, with molecular mass between 60-63 kDa. These results confirm the favorable conditions for the application of the DAT test, considering the presence of allergenic proteins suitable for immobilization and positive and negative tests, freight to the serum containing IgE specific for *D. farinae*.

The same extract was applied in the experiments using the Direct Allergy Test (DAT) method. In **Figure 2B** the proteins separated by electrophoresis (SDS-PAGE) were transferred by the sandwich-immunoblotting method. Strong staining on the nitrocellulose membrane for antigen-antibody interactions was observed after staining and the absence of color for *Trichophyton rubrum* is confirmed. The previous realization of these experiments served to confirm the presence of allergens in the extract of the *D. farinae* mite for its later application by the DAT method.

According to **Figure 3** the five experiments using the DAT method were in agreement, characterized the repeatability of the method, demonstrated by the appearance of the purple color for effective antigen-antibody binding. In a simple way the detection parameter (coloration) was obtained by the intensity of the signal versus the pixel of the image for the area of 1.53 cm^2. In **Figure 4**, negative reactivity was obtained for the DAT method when applying the proteins present in the extract of the fungus *Trichophyton rubrum* fungus as antigen.

It is observed in**Figure 5**, that there is a slight variation in the intensity of the response signal obtaining a greater signal for the addition of 2.5 mg of protein (allergens) on the glass support, and thereby intensifying the staining in the development stage. It may be considered that there is a greater availability of epitopes to occur binding with specific IgE.

V. Conclusion

From this result it can be inferred that the techniques show concordant (colorimetric) results between the classical Western blot method and Direct Allergy Test (DAT). This shows the efficiency and the advantages of using the DAT methodology because it eliminates the use of aggressive reagents such as mercaptanol and bis-acrylamide (toxic reagents) and minimizes the use of large amounts of reagents, such as the preparation of saline buffers for electrophoretic and transfer runs, requiring less analysis time, besides the reuse of the support. The Direct Allergy Test (DAT) method applied to serum tests of individuals, sensible to the Dermatophagoides farinae mite was qualitatively efficient when compared to the classic Western Blotting (immunoblotting) method. DAT is simple to execute and interpret, especially when the objective is a rapid qualitative determination of the presence of specific IgE for a certain allergen. Its application as a laboratory practice in the hospital clinic is very promising, as well as in carrying out population analyzes for large-scale studies.

Disclosures

The authors have no financial conflicts of interest.

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