
Skin prick test evaluation of *Dermatophagoides pteronyssinus* diagnostic extracts from Europe, Mexico, and the United States

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Background: Previous Food and Drug Administration (FDA)–approved enzyme-linked immunosorbent assay testing of *Dermatophagoides pteronyssinus* diagnostic extracts showed potencies of 36% to 44% for 3 European extracts relative to the FDA standard (10,000 AU/mL).

Objective: To compare biological activity of various European *D pteronyssinus* diagnostic extracts against an FDA-validated extract using quantitative skin prick tests.

Methods: Six diagnostic *D pteronyssinus* extracts (1 reference extract, which was made up of 10,000 AU/mL of the FDA-approved extract; 3 European extracts; 1 US-Mexican extract, which is imported as raw material from the United States and sold in Mexico; and 1 Mexican extract) were tested during 2 skin prick test sessions as a concentrate and 2 serial 2-fold dilutions, in quadruplicate, on the backs of 19 patients with *D pteronyssinus* allergic rhinitis. The Wilcoxon test for linked random samples was used in each group to investigate whether the distribution of the reference extract differed from each of the test extracts to a statistically significant degree (test level $\alpha = .05$).

Results: Extracts showed good dose response in wheal size for the concentrate compared with the 2 dilutions (steep part of the curve). All 3 European extracts (2-sided asymptotic significance, $P = .003$, $P = .009$, and $P = .01$, respectively) and 1 Mexican ($P < .001$) extract were less potent than the reference extract. European extracts varied in potency from 5,400 to 6,126 AU/mL, the US-Mexican extract had a potency of 7,444 AU/mL, and the Mexican extract had a potency of 2,099 AU/mL.

Conclusions: Our study confirmed the results from previous in vitro testing. Various diagnostic extracts of *D pteronyssinus* used in Europe and Mexico are less potent than those used in the United States. Similar comparisons using therapeutic extracts would be of interest.

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INTRODUCTION

Allergen extracts used in the United States may vary in biological activity when compared with those used in other parts of the world. Many immunotherapy studies performed in Europe and assays on allergy skin testing are used to guide diagnosis and treatment beyond the European borders.¹ The European influence extends into the United States² and Latin America.³ As such it has become progressively more impor-

tant to understand how the biological activity of allergen extracts from European and US manufacturers compare. The biological activity of an extract can be measured by in vitro tests or skin testing. Laboratory assays are practical; however, the results depend on the type of assay and the reagents used. At this point, there is still no universally adapted method. The CREATE (Development of Certified Reference Materials for Allergenic Products and Validation of Methods for Their Quantification) project is making good progress with the unification of in vitro testing, but until this moment only reference molecules for birch (rBet v 1), timothy (rPhl p 5a), and house dust mite (HDM) (rDer p 2) are available, together with several sandwich enzyme-linked immunosorbent assays (ELISAs) for quantitation.⁴ Because European and US mite extracts differ in their relative amounts of group 1 and group 2 major allergens,⁵ an alternative and possibly more clinically relevant way to compare European with US extracts is by quantitative skin testing because all allergic molecules present in a HDM extract contribute to the size of the wheal that is generated by this method.

In Mexico there are 3 groups of licensed manufacturers of allergen extracts: European manufacturers, manufacturers that buy the extracts as raw material in the United States and condition the extracts without standardizing for sale in Mex-

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ico, and manufacturers that mainly commercialize extracts produced in Mexico.³ In a previous study⁶ where we compared these 3 groups of extracts (European, US-Mexican, and Mexican extracts) against US extracts by ELISA inhibition testing, the tested European *Dermatophagoides pteronyssinus* diagnostic extracts showed relative potencies of 36% to 44% compared with the Food and Drug Administration (FDA) standard extract of 10,000 AU/mL (IgE competition ELISA method as recommended by the Center for Biologics Evaluation and Research of the FDA) (Table 1). In this article, we report a comparative study of the biologic activity using quantitative skin prick testing of the HDM diagnostic extract of the same 3 European manufacturers (Eur1- Eur3) and of 1 representative each of groups 2 and 3 (Table 1), called in the present study Mex1 and Mex2, respectively.

METHODS

Trial Design

The aim of the study was to compare the biological activity of European, American, and Mexican diagnostic allergen extracts. A double-blind, single-center study was undertaken. Concentrated *D pteronyssinus* skin prick test extracts and their 2 serial dilutions from 6 different manufacturers were tested in a masked random order in all patients. The study was conducted in the summer of 2008.

Patient Selection

Patients 18 to 55 years old with clinical symptoms of rhinitis (and mild asthma) were preselected as candidates for the trial from the outpatient clinic of the Centro Médico Siglo XXI, Mexico City, Mexico. Candidates had to have rhinitis symptoms on exposure to HDM, with worsening during the HDM season in Mexico. Moreover, HDM allergy was documented by a positive reaction to *D pteronyssinus* during a routine skin test with a basic panel of 18 allergens (including HDM, cat, dog, tree, grass and weed pollen, and molds; glycerinated

extracts of 1:20 wt/vol; Allerstand, Mexico City, Mexico). Finally, a negative reaction to allergen diluent with a wheal diameter less than 3 mm and the signing of the informed consent form led to inclusion in this trial. Medication that interferes with skin reactivity was suspended according to international recommendations.² Moreover, patients with proven pregnancy, patients taking psychotropic medication, patients with a positive reaction to the diluent (wheal ≥ 5 mm²), and patients who received immunotherapy in the last 5 years were excluded. The study was approved by the institutional review board of the study center (Centro Médico Nacional, Siglo XXI, Mexico City, Mexico).

Allergen Extracts

The extracts compared in this study were the glycerinated, phenolated, saline solutions of *D pteronyssinus*, commercialized as diagnostic skin prick test extracts from 3 European allergen manufacturers (all members of the European Allergen Manufacturers Group), 2 Mexican manufacturers, and 1 US manufacturer. The only European *D pteronyssinus* products included were those with license for use in human beings in Mexico: 100-HEP/mL extract (Laboratories Leti, Barcelona, Spain), extract standardized in units of biological equivalents per milliliter (IPI-ASAC, Alicante, Spain), and 30-HEP/mL extract (Soluprick; ALK-Abelló, Madrid, Spain), from here on called Eur1, Eur2, and Eur3 in a random order. The Mexican *D pteronyssinus* products tested were diagnostic extracts imported as raw material from the United States and conditioned for sale (1:20 wt/vol; Alerquim, Mexico City, Mexico) and a *D pteronyssinus* extract (1:20 vol/vol; Rocel, Puebla, Mexico), from here on called Mex1 and Mex2. The US reference extract used in this study was the FDA standardized diagnostic extract (10,000 AU/mL; Greer Laboratories Inc, Lenoir, North Carolina). Moreover, histamine (1:400 wt/vol; Alerquim) and 50% glycerin were tested as the positive and negative controls.

Table 1. Analytical Results of In Vitro Testing of Diagnostic Allergen Extracts for *Dermatophagoides pteronyssinus*, Bermuda Grass, and Cat From European and Mexican Sources^a

Extract source	Relative potency ^b of house dust mite (<i>D pteronyssinus</i>)	Relative potency ^b of Bermuda grass (<i>Cynodon dactylon</i>)	Fel d 1 content ^c in cat (<i>Felis domesticus</i>), U/L
US reference	1.000	1.000	18
Eur1	0.360	0.089	29.4
Eur2	0.444	0.246	2.1
Eur3	0.398	0.083	2.6
US-Mex1 (Mex 1 ^d)	0.411	0.287	4.4
US-Mex2	0.337	0.028	1.6
Mex1	0.082	0.090	<0.6
Mex2	0.005	0.049	1.3
Mex3 (Mex 2 ^d)	0.034	0.046	1.7

^a Adapted from Larenas-Linnemann D, Esch RE, Guidos-Fogelbach G, et al. European and Mexican diagnostic allergen extracts perform differently from US (CBER/FDA) extracts in vitro. *Allergia Immunopath*. In press.

^b Relative potency reported with respect to the US reference extract determined by IgE competition enzyme-linked immunosorbent assay (Center for Biologics Evaluation and Research, Food and Drug Administration–recommended methods).

^c Fel d 1 content determined by the Center for Biologics Evaluation and Research, Food and Drug Administration radial immunodiffusion assay.

^d US-Mex in this previous study was Mex1 in the study presented herein, and Mex3 in the previous study was Mex2 in the study presented herein.

Skin Prick Testing

All extracts were tested with an auto-click lancet by the same skin prick test technician as a concentrate and as two 2-fold serial dilutions (concentrate, dilution 1:2, and dilution 1:4) on the patient's back in 3 sessions per patient. As such, all extracts were tested in quadruplicate, in accordance with the Nordic guidelines⁷ as described in the European Academy of Allergy and Clinical Immunology position paper on allergen standardization and skin tests.⁸ The biological activity of each allergen solution was determined, tracing the wheal induced by the skin prick test on the patient's skin 20 minutes after puncture.

Masking

The extracts under investigation were bought and packed in masked vials. Personnel not involved in the study assigned numbers to all vials that included the serial dilutions of the extracts and the positive and negative controls by using a randomization list. Both the patient and the medical staff members who performed the skin prick tests were masked to the extracts, the dilutions, and the positive and negative controls. Collaborators at the statistical center were masked at the moment of data entrance. After that, it was revealed which wheals belonged to the negative and positive controls and which wheals belonged together as dilutional series of each extract. In the last step, only after all the statistical calculations were finished, the US reference extract was unmasked for the writing of this document, together with the origin of the other extracts (European or Mexican), but not the manufacturers.

Data Transfer and Statistical Analysis

Translucent tape was used to transfer the pen drawing of the wheal contour into the case report form. At the statistical center the wheal area was digitized and analyzed using the software AdobePhotoshop 6.0 (Adobe Systems Inc, San Jose, California) and Image J1.40g (National Institutes of Health, Bethesda, Maryland). The geometric mean of the patient's 4 individual wheal areas for each concentration was determined, and the log of the mean values was plotted. The 3 concentrations of each test extract were calculated based on the $\log_{10}$ wheal area and $\log_{10}$ allergen concentration for each patient.

The geometric means of the wheal areas of the test extracts were compared with the results of the US reference extract of 10,000 AU/mL and its 2 dilutions. The concentrate of the US reference extract was the comparative value for the concentrates of the test extracts (Eur1, Eur2, Eur3, Mex1, and Mex2 concentrates). To convert the geometric mean to allergy units per milliliter, a reference concentrate factor, 10,000 AU/mL, was needed. In a next step, the results of the test extract concentrates were divided by that factor to get their mean potency value. The procedure is the same for the 1:2 dilutions, using the reference 1:2 dilution factor (5,000 AU/mL). This permitted the calculation of an individual corresponding allergy unit value for the extract solutions and its dilutions per

manufacturer. The Wilcoxon test for linked random samples was used in each group to investigate whether the distribution of the US reference extract differed from each of the test extracts to a statistically significant degree with a test level of $\alpha = .05$.

RESULTS

Demographic Data of Study Patients

A total of 24 patients were recruited; 19 patients had a complete data set at the moment of analysis (range, 17-43 years; mean, 27.7 years; 9 male), giving a total of 4×19 test results for each extract dilution. One patient did not return for the second test day. Four patients were excluded in the final analysis; 2 had an incomplete case report form, and the other 2 had negative controls of 5 mm² or greater. All patients had allergic rhinitis; 6 also mild persistent asthma, with peak flow measurements greater than 80% of the predicted values on the test days. Sixteen of the 19 patients were sensitized to other allergens, apart from HDM. All had normal blood pressure at the beginning and end of the study.

Adverse Events

The adverse events reported by the investigators were all classified as certainly or probably related to the skin testing. There were 18 local reactions (39% of the tests) of intense itching on the back. One patient complained of rhinitis symptoms, which quickly faded with an oral antihistamine, but the patient did not return for the second test day. All patients were discharged 1 to 2 hours after the testing.

Skin Prick Test Results: Comparison of Absolute Wheal Sizes

The test extracts of all 6 manufacturers showed a good dose response in mean wheal area for the concentrate and its 2 serial dilutions. This finding confirms that the concentrations tested are situated on the steep part of the dose-response curve. Geometric means of the wheal area of the concentrates were as follows: reference, 3.21 cm²; Eur1, 1.96 cm²; Eur2, 1.73 cm²; Eur3, 1.91 cm²; Mex1, 2.39 cm²; and Mex 2, 0.67 cm². In Figure 1 the logarithm of the mean wheal diameters of the concentrates and both dilutions are depicted (SD of geometric means of concentrates varies between 0.5 and 2.1 cm²).

All 3 European extracts (Eur1-Eur3) were less potent than the US reference extract when tested with a 2-sided asymptotic significance: Eur1 vs US reference, $P = .009$; Eur2 vs US reference, $P = .003$; and Eur3 vs US reference, $P = .01$ (Figure 2A and B).

No statistically significant difference was seen between the US reference extract and Mex1, the extract imported as raw material from the United States. The Mex2 extract had a much lower potency, producing a wheal size 20% of that produced by the US reference. Statistically significant differences found between the European and Mexican test extract concentrates are depicted in Figure 2C.

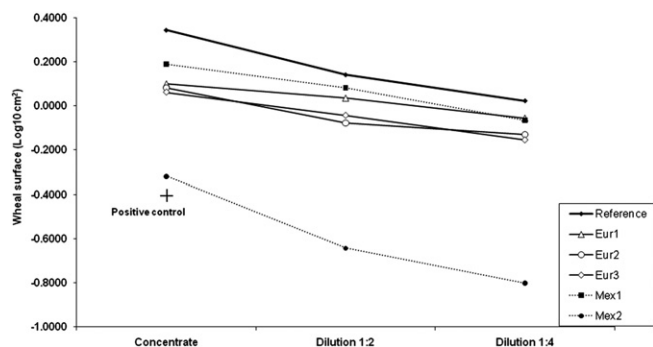


Figure 1. Log distribution of the geometric mean of the wheal surfaces of the test extracts (concentrate) and their two 2-fold dilutions (1:2 and 1:4) in comparison with the reference extract concentrate and its dilutions (bold black line). Fine continuous lines indicate European extracts; interrupted lines, Mexican extracts. For SDs, see Figure 2.

Skin Test Results: Relative Potency of All Extracts

In a further analysis, the potency of all extracts was expressed in allergy units per milliliter, relative to the US reference extract of 10,000 AU/mL. The relative potency of the European concentrate extracts was 5,400 AU/mL for Eur1, 6,127 AU/mL for Eur2, and 5,953 AU/mL for Eur3. The results for both Mexican extracts were more scattered, with Mex1 having a potency of 7,444 AU/L and Mex2 having a potency of 2,099 AU/mL (Figure 3).

DISCUSSION

In this study the biological activity of the standardized diagnostic extract of *D pteronyssinus* from a US manufacturer is statistically significantly higher than the activity of the standardized diagnostic extract of *D pteronyssinus* from 3 European manufacturers and the nonstandardized diagnostic extract from a Mexican manufacturer. The relative potency of the European diagnostic extracts of HDM is about half that of the US reference extract. The relative potency of the tested Mexican HDM diagnostic extracts varies from 20% to 70% of the US reference extract.

The concentration of a diagnostic extract has an optimal range in which there is a balance between sensitivity and specificity, and this balance may vary according to the circumstances in which the test is used. For example, the predictive value of a diagnostic reagent may vary according to the prevalence of true-positive test results in the population studied. In a specialist allergy practice, the proportion of patients with a true allergy is likely to be high, such that a potent (sensitive) extract in skilled hands is needed to exclude false-negative results, whereas for the purpose of population screening a weak (more specific) extract would be more likely to detect true allergic individuals while reducing the number of false-positive results.

Our study raises the question of which concentration would have the highest diagnostic accuracy and calls for a trial in which skin test reactivity is compared to an alternative crite-

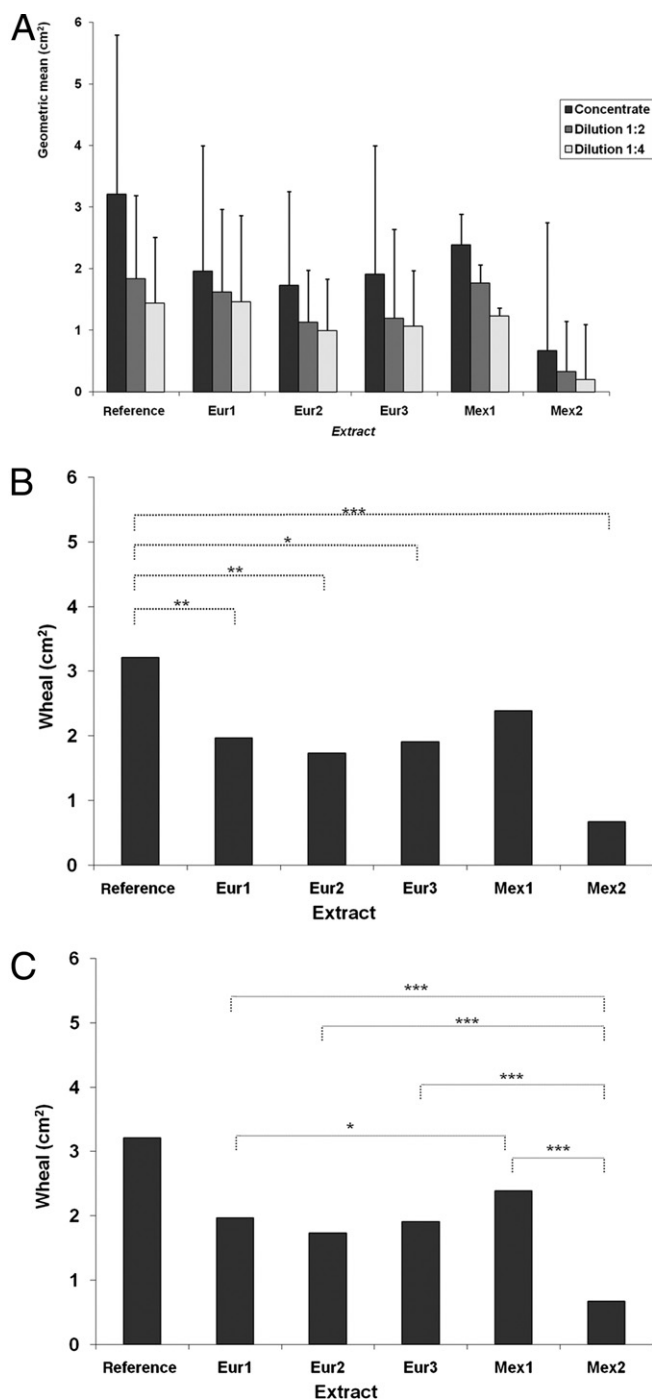


Figure 2. A, Log distribution of the geometric mean of the wheal surfaces of the test extracts (concentrate) and their two 2-fold dilutions (1:2 and 1:4) in comparison with the reference extract concentrate and its dilutions (error bars indicate SD). B, Statistically significant differences between the reference extract and the concentrate extracts are indicated as follows: * $P < .05$, ** $P < .01$, and *** $P < .001$ (Wilcoxon, 2-sided asymptotic significance). C, Statistically significant differences between the concentrate extracts of group A (European), B (US derived), and C (mostly locally made) are indicated as follows: * $P < .05$, ** $P < .01$, *** $P < .001$ (Wilcoxon, 2-sided asymptotic significance).

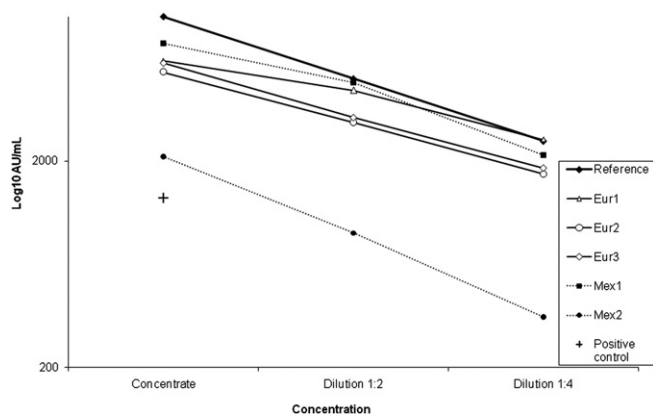


Figure 3. Log distribution of the potency as expressed in allergy units per milliliter of the concentrations of the extracts and their dilutions based on the geometric mean of the wheal surfaces of the test extracts (concentrate) and their two 2-fold dilutions (1:2 and 1:4) in comparison with the reference extract concentrate and its dilutions (bold black line). Fine continuous lines indicate European extracts; interrupted lines, Mexican extracts. Reference concentration was 10,000 AU/mL; half reference concentration, 5,000 AU/mL; and one-fourth reference concentration, 2,500 AU/mL.

rion standard of allergy diagnostic testing, such as target organ challenge tests. As stated herein, the optimal concentration may vary for different populations in different environments according to the prevalence of allergy and the diagnostic experience and skill of the operator.

Allergists in Mexico use both national and international providers for their allergens³; the results of our study have been discussed with manufacturers in Mexico, and the provider of the Mex2 extract is adjusting the concentration of its HDM diagnostic extract.

The biological activity and subsequent standardization of an allergen extract can be based on intradermal or skin prick testing. In the United States, biological standardization of extracts is performed by evaluating the erythema induced by intradermal application of the allergen.⁹ In Europe, most allergen manufacturers define the potency of their in-house reference standard extracts by wheal size by use of skin prick testing.⁷ A similar technique as used in this study comparing serial dilutions of extracts in skin prick tests has been previously applied with success to establish the relative biological activity of different therapeutic extracts for sublingual use.¹⁰ Moreover, intradermal testing was thought to be more laborious. For these reasons, we chose to use an adapted skin prick test protocol for this assay.

The present study was conducted with a single batch of all products that were used simultaneously in the same patient to reduce bias. Our stringent statistical analysis required all tests to be performed in quadruplicate. To meet this condition, all patients underwent skin tests twice. For safety reasons, we tested a maximum of 6 extracts and their dilutions in duplicate simultaneously. This biological restriction prevented testing more than 1 lot per manufacturer. We chose a single-center study because a previous multicenter study that used

skin prick testing of a standard panel of allergens revealed that in many European countries there was difficulty in unifying techniques.¹¹

A recent German study compared several timothy extracts, both diagnostic and therapeutic, from various European manufacturers with laboratory assays. In Europe only 1 manufacturer, HAL-Allergy, standardizes its products by the intradermal method used in the United States. Just as the US extract in our present study, this diagnostic extract showed statistically significant higher potency than the other extracts.¹² Few studies have compared potency of current commercially available extracts by skin testing. One assay compared 2 different European extracts by skin prick testing. In that study, SLITone solutions of birch, grass, and HDM of 1,000 STU/mL were statistically less potent than the 100-IR/mL solutions.¹⁰ No previous comparison between European and US extracts has been made. Although allergen extracts, even standardized, may vary from lot to lot, the relationship found in our study between the biological activity of European and US diagnostic *D pteronyssinus* extracts seems consistent because a similar picture was found when other lots of the same extracts were compared by various in vitro tests. In that laboratory assay, the 3 European extracts showed a relative potency of approximately 50% when tested against the US reference (*D pteronyssinus*, 10,000 AU/mL; Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland).⁶

The Mexican HDM diagnostic extract, Mex1, is prepared from raw source material bought from the same provider used

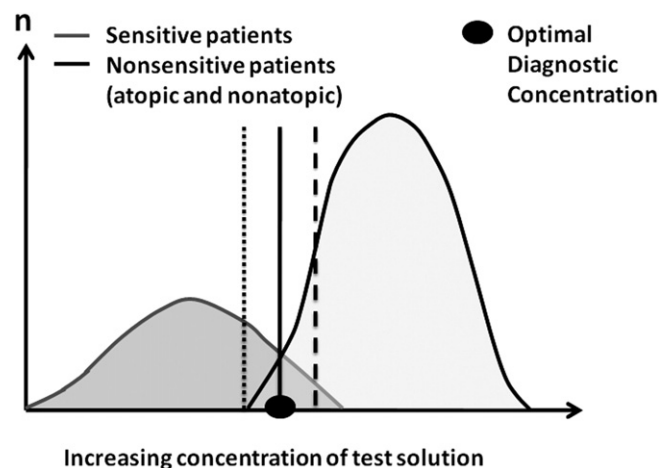


Figure 4. Allergic patients produce a wheal response at lower concentrations of the extract (light gray line) than people without allergic symptoms (dark gray line). Theoretically, if the concentration of the extract is high enough more patients may react. The optimal diagnostic concentration of an extract is around the black vertical line in the middle, leading to the lowest amount of false-positive and false-negative results. Too low concentrations of diagnostic extracts (dotted vertical line) may lead to many false-negative responses, whereas too high concentrations (interrupted vertical line) will lead to more false-positive responses.

by US manufacturers. This might explain why the biological activity of Mex1 is not dissimilar from the US reference.

With the method used in this assay of quadruplicate skin testing using 2 serial dilutions of each extract, during 2 skin prick test sessions, no severe adverse reactions occurred. The technique was simple and acceptable to patients. The dropout rate in our study was 5 of 24 patients.

Our results call for further comparisons, including allergen extracts from other prominent European manufacturers such as Allergopharma, HAL-Allergy, and Stallergènes to get a more complete European picture. Testing other allergen extracts, especially pollens and cat extracts, would also be of interest. The combination of both in vitro and in vivo comparative testing of allergen extracts used in the United States, Europe, and elsewhere will facilitate a more rational comparison of extracts used for clinical diagnosis and immunotherapy. For this purpose, the same skin prick test protocol could be used, although it must always be borne in mind that it is just a surrogate of the real biological activity that can only be tested in clinical trials. In the future, comparative studies of allergen extracts by skin testing might usefully be compared with challenge tests in the target organ, specifically nasal, conjunctival, and/or bronchial provocation tests.

An alternative approach to test patients' responses to allergens in an extract is with a multiplex system, for which only a minor amount of serum is needed. Component-resolved diagnostics with the multiplex system using serum samples from Mexican patients allergic to HDM found detectable specific IgE to Der p 1 in 53% and to Der p 2 in 50% of the patients, indicating the importance of both Der p 1 and Der p 2 and the balance of these 2 molecules in an extract (Adriano Mari, personal communication). In vitro testing should become part of the evaluation needed for the selection of an optimal diagnostic extract.

Comparing European and US HDM extracts is important because the probable effective dose for HDM subcutaneous immunotherapy, as stated in the US Practice Parameters on Immunotherapy update,² is largely based on European clinical trials. However, the doses used in these European trials were translated into allergy units based on in vitro tests but not on in vivo tests.

In conclusion, the diagnostic extracts of *D pteronyssinus* from Europe and Mexico used in our study were less potent than those from the United States. A similar comparison

using therapeutic immunotherapy extracts would be of interest. **Figure 4.**

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