Diagnostic value of serum precipitins to mould antigens in active hypersensitivity pneumonitis

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ABSTRACT: Serum precipitins have a controversial diagnostic value in hypersensitivity pneumonitis (HP). The present authors' objective was to assess their diagnostic value by developing scores from a panel of specific antigens tested by two techniques (electrosyneresis and double diffusion) to discriminate active HP from other interstitial lung diseases.

Consecutive patients presenting with a condition for which HP was considered in the differential diagnosis were included in the study. All patients underwent the same standardised diagnostic procedure, including precipitin tests performed in routine conditions. Clinical manifestations, bronchoalveolar lavage and high-resolution computed tomography defined the presence or absence of HP. Receiver-operating characteristic curves and logistic regression were used to develop the serological scores.

A total of 122 patients (including 31 cases of HP) were included in the study. Five antigens from the panel were selected for the serological scores (*Absidia corymbifera*, *Eurotium amstelodami*, *Wallemia sebi*, *Saccharopolyspora rectivirgula* and mesophilic *Streptomyces* sp.). Electrosyneresis was more discriminative than the double-diffusion technique. Predictive negative values varied 81–88% and predictive positive values varied 71–75% for prevalence of HP 20–35%.

In conclusion, serological scores using a panel of relevant antigens may guide both biological and clinical practice in areas of high prevalence of hypersensitivity pneumonitis.

KEYWORDS: Diagnosis, hypersensitivity pneumonitis, precipitins, prospective study, serology

Interstitial lung diseases (ILDs) represent a large group of diseases that includes hypersensitivity pneumonitis (HP). HP, also called extrinsic allergic alveolitis, is a syndrome that results from an immunologically induced inflammation of the lung parenchyma in response to inhalation of a large variety of antigens to which subjects have been previously sensitised. Despite its apparently low prevalence, the impact of HP in individuals of all ages throughout the world continues to be a major concern [1]. The most common forms are farmers' lung (FL) and bird breeders'/fanciers' lung (BBL). Their prevalence rates and distribution vary widely among countries and geographical locations due to local customs and occupational and climatic conditions [2]. Differential diagnosis between HP and other ILDs is difficult and relies on an array of signs or criteria [1, 3–5]. Although nonconsensual, these diagnostic indicators generally include the following: 1) clinical symptoms and signs developed in an appropriate environment; 2) the presence of precipitating antibodies against offending antigens;

3) consistent chest radiograph or high-resolution computed tomography (HRCT); 4) bronchoalveolar lavage (BAL) lymphocytosis; 5) decreased carbon monoxide diffusion capacity; and/or 6) a granulomatous reaction on lung biopsies; and/or 7) a positive inhalation challenge. Among the above-mentioned indicators, the diagnostic value of serum precipitins has long been controversial [6], particularly because of their lack of sensitivity and, especially, of specificity [7]. These limitations may be due to the use of poorly purified antigens, the lack of the specific inciting antigen in the test panel, inappropriate techniques [8] or, in epidemiological studies, the use of inappropriate control groups [5, 9, 10]. Despite the pitfalls presented above, a recent multicentre study by LACASSE et al. [4] identified serum precipitins as a significant predictor of HP, regardless of exposure (odds ratio 5.3 (95% confidence interval (CI) 2.7–10.4)). However, the choice of antigens tested and the use and interpretation of tests were left to each investigating centre according to their usual local practice; thus, the diagnostic value of precipitins could not be

assessed reliably. With the objective of developing serological scores to discriminate active HP from other ILDs, the present authors conducted a prospective cohort study of patients presenting in their department with a suspected diagnosis of HP. Subjects were tested by two serological techniques (electro-syneresis and double diffusion) for a panel of seven antigens. The choice of both immunological methods and the antigenic panel was based on previous studies aimed at identifying the best immunological set for diagnosing HP in the present study's region [11, 12].

METHODS

Patients

From January 1, 1999, consecutive patients were recruited aged ≥18 yrs presenting in the Dept of Respiratory Diseases (University Hospital of Besançon, Besançon, France) with a pulmonary syndrome for which HP was considered in a differential diagnosis (patients diagnosed in 1999 were also included in the study by LACASSE et al. [4]). The proportion of HP and other ILDs was unspecified a priori.

All patients underwent the same standardised procedure (discussed later). Only HP cases presumably due to moulds (fungi and thermophilic actinomycetes) were included, since BBL, chemical HP and hot-tub lung are due to antigens of a nature different from those of other HP caused by micro-organisms.

Diagnostic criteria

Since patients were to be classified as either HP or non-HP (other ILD), and in the absence of a recognised gold standard, the following diagnostic criteria were used for HP according to those of a previous HP study [4]. 1) Compatible clinical manifestations; 2) BAL lymphocytosis (≥30% for non- and ex-smokers and ≥20% for current smokers); and 3) bilateral ground glass or poorly defined centrilobular nodular opacities. When the association of BAL and HRCT did not yield a reliable diagnosis of HP, the presence of a positive specific provocation test [13], an improvement after avoidance of supposed antigens, or suggestive features on transbronchial biopsies was required. Patients underwent surgical lung biopsy when the previously described diagnostic procedure failed to yield a diagnosis. Classical histopathological descriptions were used [14]. Patients considered to have residual HP (a chronic and inactive form in which BAL lymphocytosis has disappeared) were classified in the non-HP group. When HP was diagnosed, the patient's exposure to antigenic sources of HP was then specifically considered in order to determine the nature of HP, e.g. FL, other mould-induced occupational HP, home HP caused by moulds, etc.

When a complete diagnostic procedure set did not lead to a reliable classification (for example, difficulties in interpreting HRCT), cases were reassessed by an independent ILD expert (J-F. Cordier), who considered, in particular, BAL and HRCT data but also data on clinical history, physical examination, spirometry and diffusing capacity of the lung for carbon monoxide (or the results of a 6-min walk test) to reach a diagnosis. If diagnosis remained inconclusive, patients were excluded.

Precipitins tests

The choice of antigenic panel was based on the recognised causes of FL and HP due to moulds at an international [15, 16] and local level [11]. The antigenic panel tested included *Absidia corymbifera, Eurotium amstelodami, Wallemia sebi, Aspergillus fumigatus, Saccharopolyspora rectivirgula*, mesophilic *Streptomyces* sp. and hay extract. Antigen extracts were produced and tested as described in a previous article [11]. The immunological methods used were agar gel double diffusion and electrosyneresis on cellulose acetate, which, as shown by a recent study, are more relevant than ELISA and immunoblots in discriminating FL from healthy exposed dairy farmers in the present study's region [12]. Briefly, electro-syneresis was carried out as follows. Cellulose acetate sheets (Sartorius, Göttingen, Germany) were placed in the electro-phoresis vat filled with buffered Tris glycin solution at pH 8.8; 15 μL of each serum was placed on three spots on the anode side and a 15-μL line of antigen was placed on the cathode side. A current (110 V) was then applied for 75 min and the sheets were stained with Coomassie blue. Serological tests were performed and interpreted (by counting the number of arcs) under routine conditions by four different experienced technicians and a supervisor blinded to the patients' diagnoses and exposure.

Statistical analysis

Characteristics of HP and non-HP patients were compared using the two-tailed Fisher's exact test for dichotomous variables and the unpaired t-test for continuous variables.

The serological results for each antigen were interpreted as a number (x_a) of precipitating arcs (discrete quantitative variable). They were used, either as discrete (x_a) or dichotomous variables (x_d), to develop serological scores. The selection of antigens for multivariate analysis was based on receiver-operating characteristic (ROC) curves to each antigen [17]. Efficiency was used to determine the positivity threshold of each antigen. Serological scores were then constructed for electrosyneresis and double diffusion using multivariate logistic regression (full model) and stepwise logistic regression (predictive model). Coefficients (β) were estimated with their 95% CIs by multivariate analysis. Scores (S_a and S_d) with antigenic results as discrete or as dichotomous variables (X_d: positive or negative) were expressed respectively, as follows.

$$S_a = \beta_0 + \sum \beta_a X_a, \text{ with } X_a = \text{number of precipitating arcs for each antigen tested} \tag{1}$$

$$S_d = \beta_0 + \sum \beta_d X_d, \text{ with } X_d = 0 \text{ (negative) or 1 (positive) for each antigen tested} \tag{2}$$

The stability of the models was studied using the bootstrap method [18]. In total, 1,000 random bootstrapped samples (size=study sample size) were generated, and a robust calculation of 95% CIs and coefficient of variation (CV) of estimated parameters (β) was obtained.

ROC curves were constructed and areas under the curves (AUC) were compared for electrosyneresis and double diffusion in order to determine the best immunological method.

Thresholds of serological score positivity for different HP prevalence were determined on ROC curves [17], and their associated predictive values were calculated. The serological

score results, expressed as either positive or negative, were then studied in association with patients' exposure and smoking status.

Estimated accuracy of serological scores

With the hypothesis of sensitivity (Se) and specificity (Sp) values between 70–90% and an HP expected prevalence of 35% (in the present authors' centre), 120 patients needed to be included in the study in order to obtain an accuracy of ~10% for Se and Sp.

RESULTS

Patients

Between January 1999 and April 2005, 162 patients were enrolled in the present study. Of these, 40 were excluded for the following reasons: BBL (n=3), hot-tub lung (n=2); unavailable or uninterpretable BAL (n=3); serological results unavailable (n=30) and indeterminate diagnosis (n=2). The independent investigator reassessed eight cases, three of which were classified in the HP group and four in the non-HP group; one was inconclusive. Thus, 122 patients were included, comprising 31 HP (22 FL, three HP due to nonprofessional farming activities (sheep and horses), two home HP due to moulds, one HP due to nonprofessional sawing (mould from sawdust), one cheese-workers' lung, and two HP with no identified exposure) and 91 non-HP ILDs (41 idiopathic interstitial pneumonia (33 idiopathic pulmonary fibrosis, three cryptogenic organising pneumonia, two acute interstitial pneumonia, two respiratory bronchiolitis ILD, one desquamative interstitial pneumonia), 18 sarcoidosis, seven drug-induced interstitial pneumonia, four pneumoconiosis, four eosinophilic interstitial pneumonia, three collagen vascular disease, three left heart failure, two histiocytosis, two neoplasic interstitial pneumonia, two infectious interstitial pneumonia, two residual form of HP and three undetermined ILD).

Patients from the HP group were significantly younger, less frequently smokers and more frequently exposed than the patients from the non-HP group (table 1).

Selection of candidate antigens for serological scores

ROC curves for *A. fumigatus* and hay extract were respectively nondiscriminative (AUC values 0.54–0.55) and uninterpretable. Therefore, threshold values for these antigens were not determined and they were not used in multivariate analysis to develop serological scores. Positivity thresholds for each antigen for the two immunological methods are indicated in table 2.

Development of serological scores

ROC curves associated with electrosyneresis scores were more discriminative than those associated with double diffusion, regardless of the type of variables (fig. 1). Similarly, full models appeared to be more relevant than predictive models. Only results concerning electrosyneresis with the full model are presented. Estimated coefficients used to calculate serological scores and their CIs are indicated in table 3. Coefficients of variation from bootstrap re-sampling showed a moderate stability of estimated parameters (table 3).

Performances of serological scores

The positivity thresholds of serological scores according to different prevalence rates (20, 25 and 35%) of HP and their diagnostic values for electrosyneresis are presented in table 4. Performances of scores with discrete variables were equivalent to those with dichotomous variables for negative predictive

TABLE 1	Description of patients' characteristics		
	HP %	Non-HP %	p-value
Patients n	31	91	
Age yrs	52.7±14.3	62.4±15.4	0.003[#]
Sex			
Male	19±61.3	54±59.3	1.0[¶]
Female	12±38.7	37±40.7	
Exposure to mould and actinomycetes			
Yes	29±93.5	19±21.1	<0.0001[¶]
No	2±6.5	71±78.9	
Tobacco exposure			
Nonsmoker	27±87.1	47±52.8	0.0006[¶]
Smoker	4±12.9	42±47.2	
Current smoker	0±0.0	9±10.1	
Ex-smoker	4±12.9	33±37.1	

Data are presented as mean±SD, unless otherwise stated. HP: hypersensitivity pneumonitis. [#]: Unpaired t-test; [¶]: Fisher's exact test.

TABLE 2	Cut-off values of serological results (number of precipitating arcs) by antigen and immunological techniques	
	Antigens	Cut-off
Double diffusion		
	Absidia corymbifera	1
	Eurotium amstelodami	1
	Wallemia sebi	1
	Aspergillus fumigatus	ND
	Saccharopolyspora rectivirgula	1
	Streptomyces mesophilic	1
	Mouldy hay extract	ND
Electrosyneresis		
	A. corymbifera	1
	E. amstelodami	2
	W. sebi	2
	A. fumigatus	ND
	S. rectivirgula	1
	Streptomyces mesophilic	2
	Mouldy hay extract	ND

ND: nondiscriminative antigen. To transform serological results from number of arcs (discrete variable) to positive or negative (dichotomous variable) using this table, serological results must be coded as 0 (negative) if the number of arcs is below the cut-off value, and as 1 (positive) if the number of arcs is equal to or above the cut-off value.
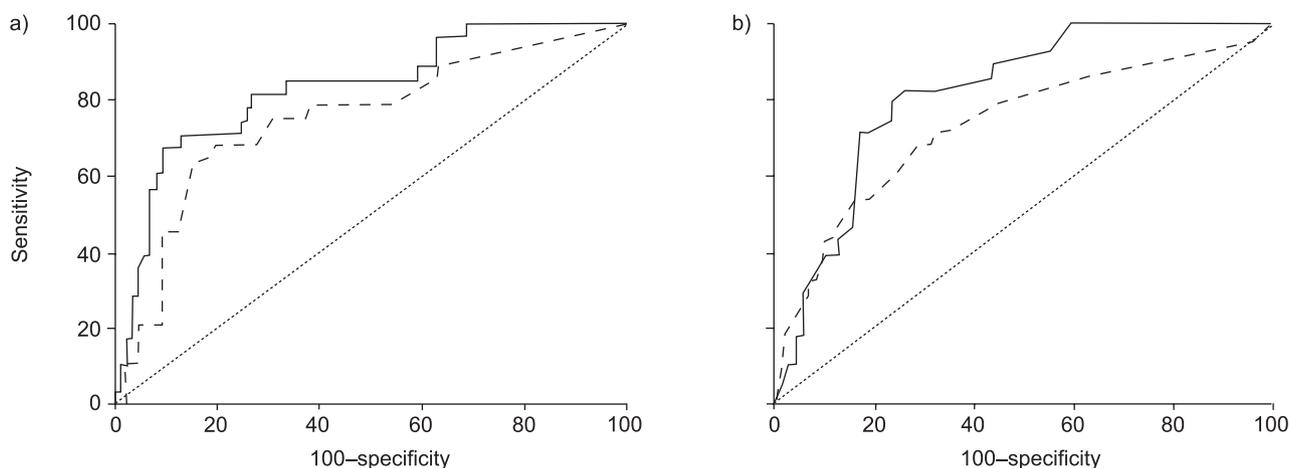
FIGURE 1. Receiver-operating characteristic (ROC) curve for electrosyneresis and double-diffusion showing a) discrete variables, full model (p=0.247; area under the curve (AUC) with electrosyneresis (Ae)=0.830; AUC with double diffusion (Ad)=0.751) and b) dichotomous variables, full model (p=0.260; Ae=0.814; Ad=0.730). The AUC is equivalent to the probability that a patient drawn randomly from the HP group has a greater score value than a patient from the non-HP group. p: Probability of the hypothesis that the difference between the two AUCs compared is 0. ——: electrosyneresis; – – – –: double diffusion.

values (NPV; 81%–88%) but better in terms of positive predictive values (PPV). In full models, PPV varied 71–75% with discrete variables and 57–64% with dichotomous variables. If serological tests were considered as positive when at least one of two scores was positive, S_p did not change but S_e was higher. For a HP prevalence of 35%, S_e was 76% (95% CI 0.60–0.92), S_p 82% (95% CI 0.74–0.90), PPV 69.5 and NPV 86.4.

After adjustment by disease group, a positive serological result, with discrete variables, was positively associated with exposure (p=0.032). However, 20% of subjects with precipitins had no identified exposure and 25% of subjects without precipitins had exposure. The presence of precipitins tended to

be negatively associated with tobacco consumption (p=0.08; table 5).

Eventually, eight false positives (8.8%) and 11 false negatives (33.3%) were obtained. The false positives were as follows. Three idiopathic pulmonary fibrosis (nonexposed), one eosinophilic interstitial pneumonia (exposed farmer), one silicosis (exposed), one left heart failure (nonexposed) and two residual FL (both exposed farmers). The false negatives comprised eight FL, one home HP due to moulds in the walls, one HP due to nonprofessional farming activities (horses) and one HP without identified exposure. The home HP subject was tested for the antigens corresponding to the actual exposure, identified

TABLE 3 Estimated parameters of serological scores with electrosyneresis for full logistic models

Antigens	Coefficient β	95% CI	p-value	95% CI[#]	CV[#] %
Discrete variables					
Intercept	-2.45				
Saccharopolyspora rectivirgula	0.61	0.04–1.19	0.04	-0.17–1.39	65
Wallemia sebi	0.33	-0.02–0.68	0.06	-0.20–0.86	83
Absidia corymbifera	0.32	-0.26–0.90	0.28	-0.57–1.21	141
Eurotium amstelodami	0.16	-0.26–0.58	0.47	-0.45–0.76	197
Streptomyces mesophilic	0.14	-0.37–0.64	0.60	-0.62–0.89	282
Dichotomous variables					
Intercept	-2.44				
Eurotium amstelodami	1.04	-0.05–2.12	0.06	-0.33–2.40	67
Absidia corymbifera	0.97	-0.04–1.97	0.06	-0.22–2.15	62
Wallemia sebi	0.72	-0.34–1.78	0.18	-0.75–2.20	104
Streptomyces rectivirgula	0.61	-0.38–1.61	0.23	-0.63–1.86	104
Streptomyces mesophilic	0.55	-0.58–1.68	0.34	-1.16–2.26	159

CI: confidence interval; CV: coefficient of variation %=β/$S_β$ × 100, with $S_β$=standard error. [#]: 95% CI and CV calculated with bootstrap. To interpret this table a patient with, for example, *S. rectivirgula*=2 arcs, *W. sebi*=0 arcs, *A. corymbifera*=1 arc, *E. amstelodami*=3 arcs and *S.* mesophilic=0 arcs, has the following score with discrete variables: S_n= -2.45+0.61 × 2+0.33 × 0+0.32 × 1+0.16 × 3+0.14 × 0= -0.43, and the following score with dichotomous variables: S_d= -2.44+0.61 × 1+0.72 × 0+ 0.97 × 1+1.04 × 1+0.55 × 0=0.18. To transform a discrete variable into a dichotomous variable, see table 2.

TABLE 4	Cut-off values (determined with the optimal criterion method[#]) and performances of serological scores with electrosyneresis					
Variable	Prevalence %	Positive serology	S_e	S_p	PPV	NPV
Discrete	20	>-0.35	49	95	71	88
	25	>-0.44	55	93	72	86
	35[¶]	>-0.53	61	89	75	81
Dichotomous	20	ND				
	25	>-0.76	60	85	57	86
	35[¶]	>-0.78	66	80	64	81

S_e: sensitivity (probability of a positive test outcome in a hypersensitivity pneumonitis (HP) individual); S_p: specificity (probability of a negative test outcome in a non-HP individual); PPV: positive predictive value (probability of having HP when serological score is above the cut-off value); NPV: negative predictive value (probability of having HP when serological score is equal to or below the cut-off value); ND: nondiscriminative. [#]: $S=((1-P)/P)((C_{FP}-C_{TN})/(C_{FN}-C_{TP}))$, where S is the slope of the receiver-operating characteristic curve at the optimal operating point, in which ''optimal'' is in terms of minimising costs (C). ''Costs'' can be identified here as patient morbidity, where C_{FP}, C_{FN}, C_{TN} and C_{TP} represent the costs of false-positive, false-negative, true-negative and true-positive results, respectively. Here, it was assumed that $C_{FP}=C_{FN}$ and $C_{TP}=C_{TN}$. Therefore, here $S=((1-P)/P)$, where P denotes the prevalence in the target population. [¶]: prevalence in study population. To use this table for a given patient from, for example, a department of respiratory disease where the prevalence of HP is nearly 35%, with a serological score of -0.43 (>-0.53) with discrete variables and 0.18 (>-0.78) with dichotomous variables, both serological scores indicate a diagnosis in favour of HP. To calculate scores, see table 3.

by microbiological sampling in the subject's home. This additional investigation showed at least two arcs of precipitation with electrosyneresis for *Fusarium verticilloides*, *Cladosporium* sp. and *Trichoderma* sp. The same investigations were carried out for the nonprofessional farming HP female and *Saccharomonospora viridis* was identified as the probable responsible antigen (four arcs of precipitation). Six out of the eight false-negative FL results had at least two arcs for hay extracts and a significant number of arcs with electrosyneresis for at least one of the present study's panel of antigens (without reaching a positive score), and the presence of 1–3 arcs of precipitation for at least two of the following antigens that were not in the present study's panel: *Aspergillus nidulans*, *Streptomyces griseoflavus*, *Thermoactinomyces vulgaris*, *S. viridis*, *Rhodotorula rubra*, *Fusarium solani* and *Penicillium* sp.

TABLE 5	Serological results of patients with electrosyneresis[#] according to exposure and tobacco consumption			
	Positive serology	Negative serology	p-value	p-value[¶]
Patients n	26	87		
Exposure				
Yes	21 (80.8)	22 (25.6)	<0.0001	0.032
No	5 (19.2)	64 (74.4)		
Tobacco				
Smoker	3 (11.5)	40 (47.0)	0.001	0.08
Nonsmoker	23 (88.5)	45 (53.0)		

Data are presented as n (%), unless otherwise indicated. [#]: Full model with discrete variables (see table 3), with cut-off associated with a prevalence rate of 35% (table 4); [¶]: Cochran–Mantel–Haenszel adjusted to Chi-squared (adjusted by disease group).

DISCUSSION

In the absence of standardised and validated diagnostic criteria, diagnosis of HP is difficult [1]. Several proposals for diagnostic criteria have been published [19–22] but their diagnostic accuracy has not been tested. Among these criteria, serology has long been considered to be either a minor criterion or a simple marker of exposure [23–26]. However, with typical FL patients, the use of antigens isolated from patients' environments has lead to convincing results [11, 27]. In addition, a recent study by LACASSE *et al.* [4] identified serum precipitins as a significant predictive factor of HP, regardless of exposure. However, the diversity of antigens and immunological techniques used in this multicentre study did not allow evaluation of the real diagnostic value of precipitins. The HP cohort in the study of LACASSE *et al.* [4] consisted mainly of BBL disease and subjects were generally tested by ELISA. In the present study, which concerns HP due to moulds, the sera of patients were tested by electrosyneresis and double diffusion using a panel of antigens that included *A. corymbifera*, *E. amstelodami*, *W. sebi*, three antigens responsible for FL in the region of the present study and rarely considered in other studies. The use of local antigens limits the feasibility of a multicentre study, and it is for this reason that the number of cases in the present study is limited.

The results of the present study showed that it is possible to use a serological score in the diagnosis of HP caused by moulds for patients suffering from an ILD, when an appropriate panel of antigens is used. The results also showed electrosyneresis to be more appropriate than double diffusion for differential diagnosis of HP. However, the sensitivity of the serological test was somewhat disappointing, since about one-third of HP had negative results. However, in eight of the 11 false-negative results for the present study's serological score, the responsibility of antigens not included in the tested panel was either suggested or demonstrated by further antigenic testing. The lack of sensitivity in scores can therefore be explained by the limited panel tested with respect to the great

diversity of antigens that cause HP. The use of a larger panel of antigens would improve the sensitivity of the methodological approach.

The most discriminative antigens within the tested panel were *S. rectivirgula, A. corymbifera, E. amstelodami* and *W. sebi*; the first is recognised as the major aetiological antigen of FL [27] and is responsible for other professional HP due to actinomycetes. The three others are likely to be aetiological factors of FL in Eastern France [11]; however, *A. corymbifera* and *W. sebi* have also been shown to be aetiological agents in FL in Scandinavia [28, 29]. Conversely, the present study, as well as the Scandinavian studies [28, 29], have not demonstrated the ability of *A. fumigatus* to discriminate between HP and non-HP. This antigen has been evoked as an aetiological factor of HP for more than four decades [30] but its actual responsibility in HP is probably rare.

One concern of the present authors is the applicability of the results of the current study. Both the panel of antigens used and the score are not strictly applicable worldwide, mainly because the current ILD cohort includes a high proportion of HP cases that do not represent the usual aetiologies of HP. However, the approach used, which consisted of developing a serological score through use of a combination of local and international antigens, can be applied worldwide. In addition, it would be useful to test the current panel in other parts of the world. Indeed, this panel probably allows detection of immunological responses induced by a larger number of fungal species and thermophilic actinomycetes, given the known cross-reactivity between different fungal species. In this respect, the present study partially answers the need to develop panels of standardised antigens, highlighted in the recent recommendations of the National Heart, Lung and Blood Institute/Office of Rare Diseases workshop [1].

Conclusion

Serological tests using a panel of relevant antigens and performed by electrosyneresis help to diagnose mould-induced hypersensitivity pneumonitis by providing precise estimates of the probability of active disease in a region where hypersensitivity pneumonitis, especially farmers' lung, is frequent.

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