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Exposure Assessment Tools for Hypersensitivity Pneumonitis: An Official American Thoracic Society Workshop Report

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ONLINE DATA SUPPLEMENT

Supplementary Material

Immunoglobulin Measurement Methods

Several laboratory methods for immunoglobulin measurement are available. Qualitative methods (e.g., double diffusion [DD], electrosyneresis [ES], immunoelectrophoresis [IEP]) evaluate precipitation of protein-immunoglobulin complexes ("precipitins") on a plate or gel agar after mixing the serum and antigen of interest, followed by visual assessment of the presence and number of precipitation bands/arcs. Quantitative methods (e.g., ELISA, ImmunoCAP) measure the quantity of serum immunoglobulin after adding serum to an antigen-coated plate, with antigen-IgG complexes detected using an enzyme- or fluorochrome-linked antibody. Quantification is done by comparing fluorescence/coloration produced by the sample to a standard curve using a mixture with known quantity of IgG, or by reporting absorbance or optical density. Qualitative methods require manual setup and take several days to complete, while quantitative methods can be fully automated and completed in several hours. A disadvantage of quantitative methods is high test-to-test variability due to the quality or purity of the antigen used. Use of either method requires selection of a threshold number of precipitation arcs or quantity of IgG considered as positive to be applied as a diagnostic test.

Table S1

Author Year; Study Location	Population Enrolled	IgG Method	Antigen*	Positive Test Threshold	Se	Sp	Comments
Recognized Bird Exposure (Pigeon unless otherwise stated)							
Anderson 1982; Denmark	Exposed, with symptoms meeting HP threshold (n=4) Unspecified exposure, healthy donors (n=85)	DD ELISA	Whole PS PDE Whole PS PDE	DD: >0 bands ELISA: Titer at least 10240	100% 100% 100% 75%	100% 100% 100% 100%	Se/Sp are for HP vs healthy blood donors. Symptoms via a questionnaire. The study also reports findings on n=51 exposed individuals with symptoms but unclear HP status, and n=89 exposed asymptomatic individuals. Given inability

							to classify some of these as HP vs not HP, calculations using these were omitted.
Baldwin 1998; England	Exposed, with symptoms (n=91), Exposed, asymptomatic (n=159) Unexposed controls (n=40)	IEP	PD, PS	IEP: >0 precipitin bands to both PD and PS	53%	51%	Precipitin results not given for the controls; sens/spec are reported for precipitin test in those with vs without symptoms. Symptoms via a questionnaire.
Rodrigo 2000; Spain	Exposed, HP (n=17) Exposed, asymptomatic (n=11)	ELISA PB	PS PB	>97.5th percentile of n=73 unexposed	100% 100%	55% 45%	Pigeon breeders with HP were identified as diseased because they had positive precipitins by IEP method. Exact threshold value for PS and PB was not specified.
Rouzet 2017; France	Exposed, HP (n=25) Exposed, pulmonary disease (n=20) Exposed, healthy (n=10)	ELISA DD+IEP	PD Extract IGLL1 ProE Crude PD	ELISA: Not explicitly stated >1 band for DD and >2 band for IEP	72% 76% 84% 60%	93% 100% 80% 93%	Diseased subjects were screened for presence of precipitin arcs to bird dropping prior to inclusion in this study. Antigens were developed using an immunoproteomic approach: proteins extracted from PD were tested against a subset of the HP and exposed healthy. Proteins with reacting IgG in HP only were tested as potential disease-specific antigens (IGLL1 and ProE). Se/Sp are for HP vs all other exposed (n=30). Positive test thresholds developed using ROC analysis on the test data.
Sandoval 1990; Mexico	Exposed, HP (n=27) Unexposed alternative ILD (n=29) Unexposed non-ILD illness (n=60)	ELISA	PS extract	>5 SD above 30 healthy blood donors.	93%	59%	Se/Sp shown are for HP vs non-HP ILD. The specificity using unexposed individuals hospitalized with unspecified illnesses is 98%.
Simpson 1992; Northern Ireland	Exposed, HP (n=50) Unexposed non-ILD pulmonary disease (n=50) Unexposed healthy controls (n=50)	ELISA DD		0.082 OD Not specified	100% 80%	100% 100%	Se/Sp shown are for exposed with HP vs unexposed with non-ILD pulmonary disease. All unexposed healthy controls had negative ELISA and DD measurements (Sp 100%). ELISA threshold based on 3 SD of the 50 healthy control serum.
Suhara 2015; Japan	Exposed, chronic bird HP (n=35) Unexposed, alternative ILD (n=76) Healthy controls (n=unspecified)	ELISA	PDE	>0.305 (units not specified)	34%	87%	Also reported Se/Sp in acute bird HP vs acute summer-type HP. Threshold for positive index selected via ROC analysis using the same samples on which sens/spec are given.
Recognized Mold Exposure							
Aznar 1988; France	Exposed farmer, HP (n=10) Unexposed controls (n=10)	Immunoblot ELIEDA	*M. faeni* extract	>0 Arcs >0 Arcs	100% 100%	100% 100%	Subjects were selected because they were known to have precipitin bands by IEP and ES methods. This study characterized presence of antibody classes and fractions by alternative precipitation methods.
Barrera 2014; France, Switzerland	Exposed farmer, HP (n=41) Exposed farmer, healthy (n=43)	ELISA	Protein panel: SR1FA, SR17, SR22 (see notes)	Not explicitly stated	83%	77%	Serology was used in the diagnostic criteria for HP. Preliminary experiments in n=4 HP and n=9 controls identified *S. rectivirgula* immunoreactive proteins specific to disease. Antigens used were developed using an immunoproteomic approach. Threshold for positive index selected via ROC analysis using the test samples.
Boiron 1987; France	Exposed bagasse workers, HP (n=26), Exposed bagasse, asymptomatic (n=19) Unexposed healthy blood donors (n=10)	IEP ELISA	*T sacchari* extract	IEP: >0 precipitin bands ELISA: Not stated	58% 88%	37% 26%	All unexposed subjects were negative by both methods (therefore specificity 100% for comparison of exposed groups to unexposed)
Fenoglio 2007; France	Mold-exposed HP (n=31) Unspecified exposure, non-HP ILD (n=91)	ES	Panel of mold extracts	Serologic score > -0.78	65%	80%	Panel includes *A. corymbifera, E. amstelodami, W. sebi, S. rectivirgula*, *Streptomyeces* sp. *A. fumigatus* and hay extract discriminated HP from other ILD very poorly and were excluded. Serologic score developed via logistic regression,

							threshold considered positive identified based on ROC analysis using the test samples. Thresholds at which each antigen were considered positive are not explicitly stated.
Hebert 1985; Canada	Exposed farmer, HP (n=29) Exposed farmer, asymptomatic (n=91) Unexposed controls (n=23)	DD	*M. faeni* extract	DD: >0 bands when serum was concentrated up to 4-fold	48%	60%	Se/Sp are for DD in exposed diseased and exposed non-diseased; all unexposed controls were negative (Sp=100%). ELISA results also reported as mean (SD); quantitative IgG was higher in the setting of disease and positive precipitin bands.
Huizinga 1985; Netherlands	Exposed symptomatic farmer, HP (n=18) Exposed symptomatic farmer, not HP (n=19) Unexposed controls (n=29)	ELISA DD	*M. faeni* extract	ELISA: Not stated; see notes. DD: >0 precipitation band	100% 100%	84% 89%	Shown here are results of ELISA and DD in symptomatic farmers with vs without HP. For HP vs unexposed controls via ELISA, Se=100% and Sp=100%. IgG-ELISA thresholds: Those with disease had IgG-ELISA >0.3 OD. Those without disease considered negative had <0.15 OD; among 3 false positives, OD measurement was not explicitly stated.
Reboux 2007; France	Exposed farmer, HP (n=15) Exposed farmer, asymptomatic (n=15) Unexposed controls (n=30)	ES DD ELISA	*A. corymbifera* extract	>1 bands >0 bands >214 absorbance	87% 73% 47%	100% 93% 87%	Authors considered *A. corymbifera* to perform best using ES method, and is reported here. *W. sebi, E. amstelodami, and S. rectivirgula* were also tested. Se/Sp are reported for the thresholds selected via AUC analysis on the same samples.
Roussel 2010; France, Switzerland	Exposed farmer, HP (n=17), Exposed farmer, asymptomatic (n=40) Unexposed controls (n=20)	ELISA	*E. amstelodami* ascospore	>0.113 OD	71%	88%	Antigen to the conida and hyphae units were also tested, with lower specificity. Se/Sp for HP vs unexposed controls are: 71%/100%. Antigens were developed using an immunoproteomic approach. Threshold identified using AUC analysis in these test samples.
Tillie 2011; France	Exposed metal working fluid, HP (n=10) Exposed metal working fluid, no HP (n=12)	ELISA ES	*M. immunogenum*	>1.6 AU >4 bands	70% 90%	92% 100%	A total of 13 subjects suspected of having disease but HP was only confirmed in n=10, which was used for Se/Sp calculation presented here. Thresholds of AU/bands considered positive was via AUC analysis in these test samples.
Mixed or Unrecognized Exposures							
Lacasse 2003; Multinational	ILD for which HP was considered in the differential diagnosis; various exposures: HP (n=116) Other ILD (n=284)	ELISA or ES – per enrolling center	Variable – per enrolling center discretion	Variable – per enrolling center's pre-specified thresholds	78%	69%	While IgG results were not part of the diagnostic criteria for this research study, measuring IgG was at the discretion of the enrolling center physician and likely influenced categorization as HP vs not HP.
Morell 2013; Spain	Patients meeting 2011 ATS criteria for IPF: HP (n=20) IPF (n=26)	ELISA	Most subjects tested for mold and bird antigens	Not stated	90%	62%	Se/Sp was extracted from supplemental Tables 1 and 2. When testing was negative or indeterminate, it was considered negative. If subject tested positive for any antigen, it was considered positive.

*When multiple measurement methods and/or antigens were tested, the antigen with the optimal sensitivity and specificity combination is reported in this table and measurement method and antigen name listed first in the respective columns.
PS: pigeon serum; PDE: pigeon dropping extract; ELISA: enzyme linked immunosorbent assay (quantitative method); EIA: enzyme immunoassay (quantitative method); DD: double diffusion (precipitation method); IEP: Immunoelectrophoresis (precipitation method); ES: Electrosyneresis (precipitation method); ELIEDA: enzyme-linked immune-electro-diffusion assay (a precipitation method which can identify immunoglobulin fractions with different antigen specificities). Se=sensitivity, Sp=specificity.

Serum Specific Immunoglobulin Measurement (SS-IgG)

As summarized in **Table S1**, two of these studies employed testing for antibodies to a variety of antigens in patients with ILD and various suspected exposures(1, 2), one tested for SS-IgG to pigeon serum extract in patients with bird-related HP and non-HP ILD(3), and one employed a panel of mold extracts among patients with mold-related HP and non-HP ILD(4). Each study used a different method for measuring IgG, and only one(3) explicitly reported the threshold for defining the test as positive, making broad application of one validated panel difficult in practice.

clinical practice. First, no two studies employ the same Ag, measurement method, or threshold for a positive test in more than one cohort of patients; therefore, no set of testing conditions has been externally validated. This is particularly problematic since many studies selected the threshold for a positive test using ROC analysis on the same set of subjects for which the diagnostic test characteristics are reported (see **Table S1** comments). When a pre-specified threshold for a positive test was used(1, 3, 5-10), the specificity was low at 37-69% after excluding two outliers with specificity 89% and 100%(8, 10), indicating a high rate of false-positive tests. Additionally, only three studies definitely(2, 4) or probably(1) tested each subject using a panel of multiple antigens. Specificity was relatively lower in these studies at 62%, 69%, and 80%.

Skin Testing

Skin testing is not commonly used for HP diagnosis or exposure characterization, and relevant articles on the topic were published before 1991(53-55). To perform skin testing, antigens are generated using extraction methods similar to SS-IgG measurement and introduced into the skin via prick or intradermal injection. The test should be read after a short (15 minutes) post-injection intervals and interpreted based on the reaction size. For intradermal *M. faeni* extract, a wheal >10mm diameter at 48 hours post-injection was found to have 50% sensitivity of and 86% specificity in distinguishing HP cases from exposed asymptomatic farmers(11). Similarly, intradermal injection of avian droppings extract had 52% sensitivity and 100% specificity at 48 hours(12). Among bird-exposed individuals, the exuberance of the skin reaction correlated with SS-IgG levels but not total serum IgE levels, suggesting that a positive skin test does not merely reflect atopy(13). Challenges with Ag extract preparation and lack of standardization limit the utility of skin testing as either an Ag identification or diagnostic tool for HP.

Lymphocyte Proliferation Testing

Few studies report results of LPT using cells harvested from BAL. In one study, seven patients with feather duvet lung were evaluated, four with acute and three with chronic disease. Blood LPT was positive in four of seven patients, and of the three patients who underwent BAL, BAL LPT was positive in two(14). A larger study found high sensitivity and specificity of BAL LPT in patients with acute bird-related HP, while blood LPT from the same patients had low sensitivity but high specificity(15). In chronic bird-related HP, the sensitivity and specificity of BAL LPT were 46% and 91% respectively(15).

Most LPT studies focus on one or two suspected antigens based on knowledge of current or past exposure(s) of interest, limiting generalizability to individuals without known antigen. However, one study investigated LPT in individuals with pigeon breeder's disease and in a group of healthy volunteers using 15 different antigenic determinants fractionated by polyacrylamide gel electrophoresis(16). A wide variety of responses was observed, with no consistent patterns in either group. Most healthy subjects responded to some soluble fractions, while patients with pigeon breeder's disease displayed the strongest responses to a significantly higher number of antigenic fractions(16). In a study of epoxy resin workers, blood LPT was explored as a biomarker of exposure and sensitization in two groups, exposed vs unexposed(17). No significant difference was found in 'abnormal' LPT between groups, reflecting challenges of utilizing LPT as a tool for identifying antigens of interest, including determination of appropriate concentrations of Ag adequate to induce a proliferative response(17).

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