

The Use of Enzyme-Linked Immunosorbent Assay (ELISA) in the Diagnosis of Farmer's Lung

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Enzyme-linked immunosorbent assay (ELISA) was adapted to measure antibodies against *Aspergillus fumigatus*, *Thermoactinomyces vulgaris* and *Micropolyspora faeni*. Mycelial and culture fluid antigens were used separately to analyze antibodies in three study groups: farmer's lung patients, patients with bronchitis and healthy control persons. The mycelial antigen of *A. fumigatus* gave more positive results than the corresponding culture fluid antigen. The situation was reversed for the actinomycetes. For this reason either a mycelial antigen or a combination of mycelial and culture fluid antigens should be used in ELISA in routine tests. The prevalence and the titers of antibodies against the three microbes detected by ELISA were higher in the farmer's lung group than in the other two groups.

Key words: ELISA; farmer's lung

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Two thermophilic actinomycetes *Thermoactinomyces vulgaris* and *Micropolyspora faeni*, and fungi of the genus *Aspergillus* are considered to be the most important microbes associated with farmer's lung syndrome (2, 9, 12, 14). The presence of circulating antibodies against these microbes has been used as supporting evidence in the diagnosis of the disease. The traditional technique to detect these antibodies has been immunoprecipitation. It is, however, an insensitive and a poorly quantitative test and alternative methods have been sought.

Enzyme-linked immunosorbent assay (ELISA) has several advantages compared with other serological techniques and in recent years it has been adapted to measure antibodies to a variety of antigens (1). It has been used to detect antibodies to *A. fumigatus* in aspergillosis patients (5, 10) and we have previously shown that it can be used to measure antibodies to *A. fumigatus* in farmer's lung patients (8).

In the present study we have expanded the use of ELISA to measure antibodies to other antigens associated with farmer's lung. Sera of farmer's lung patients, patients with bronchitis and healthy control persons were tested with both mycelial and extracellular antigens of *T. vulgaris*, *M. faeni* and *A. fumigatus* and the antibody findings compared with the clinical condition.

MATERIAL AND METHODS

Antigens

Antigens were prepared from *Thermoactinomyces vulgaris*, *Micropolyspora faeni* and *Aspergillus fumigatus*. The microbe strains were obtained from the Mycological Reference Laboratory, London. They were grown in synthetic medium VII (11) for 3-4 days. The actinomycetes were cultivated at 55°C and the fungus at 37°C. The microbial cultures were centrifuged at 1,000 × g for 20 min. The

extracellular material in the supernatant was concentrated against polyvinylpyrrolidone K 25 (50 $\times$) and dialysed for 24 h against phosphate buffered saline, pH 7.0 (PBS). The concentrate was used as the culture fluid antigen.

The mycelial pellet was washed twice with PBS. The cells were disrupted mechanically (Ultra Turrax, Janke and Kunkel, Staufen i Breisgan, FRG) and then by ultrasonic treatment (MSE 1174 MK2, Crawley, U.K.). The supernatant after centrifugation at 1,000 g for 20 min was used as the mycelial antigen. Both culture fluid and mycelial antigens were used separately in the tests. All antigens were used in ELISA at protein concentrations of 50–100 μ g/ml.

Study groups

The material of the study comprised three study groups, which were formed of in-patients of the Department of Pulmonary Diseases of the University Central Hospital of Kuopio (6, 8) and of persons interviewed in a survey for farmer's lung and chronic non-specific lung diseases (7). The farmer's lung group consisted of 17 patients with clinically proven farmer's lung, described in detail previously (6, 8). Patients with cough and sputum production at least 3 months each year for 2 consecutive years or with intercurrent episodes of severe bronchitis were included in the bronchitis group (18 patients). None of the patients in this group had any symptoms or signs suggesting farmer's lung. They were all living in a rural area, 72% being farmers or farm workers. Twenty persons, totally free of respiratory symptoms, were used as controls. They were rural inhabitants, not having been engaged in farming in the 4 years preceding the study.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed on microtiter plates (Microtiter M 29 A, Sterilin, Teddington, U.K.) according to Voller et al. (13). The conjugate was horseradish peroxidase

conjugated anti-human IgG (Orion Diagnostica, Finland). The substrate solution contained *o*-phenyldiamine, 3 mg/ml (Koch-Light Lab. Ltd) and urea hydrogen peroxide (BDH chemicals Ltd), corresponding to 0.02% H_2O_2 in 0.1 M citrate-phosphate buffer pH 5.5.

Three dilutions of each serum were tested in ELISA. The absorbances were measured with a spectrophotometer (Hitachi, Perkin Elmer 139, Japan). The absorbance curves were drawn on a logarithmic scale of the serum dilutions. The reactivity, i.e. the titer of a serum, was expressed as the negative logarithm of the serum dilution giving an absorbance of 0.5.

A positive control serum as well as a serum which was considered negative to all antigens were included in every test. The mean titer of the negative reference serum was calculated from seven consecutive tests to all antigens. The mean titers ($\pm$ s.d.) were 3.9 ± 0.2 for *A. fumigatus*, 4.3 ± 0.3 for *T. vulgaris* and 4.1 ± 0.3 for *M. faeni* mycelial antigen, and 3.2 ± 0.25 , 2.9 ± 0.25 and 3.2 ± 0.25 for the corresponding culture fluid antigens. Serum titers exceeding the mean of the negative serum by two standard deviations were considered positive.

RESULTS

The ELISA-titers of the sera determined as described in Methods are presented in Fig. 1. Titer distributions in the three study groups showed that sera of the farmer's lung patients reacted generally at higher dilution against all antigens than sera of patients with bronchitis or sera of the control persons. Comparison of titer distribution against mycelial and culture fluid antigens indicated that the mycelial antigen of *T. vulgaris* showed a larger difference in titers between the farmer's lung group and the control group than the corresponding culture fluid antigen. The titers obtained with mycelial and culture fluid antigens of *A. fumigatus* and *M. faeni* had distribution patterns similar to each other.

The ELISA-titers were divided into positive

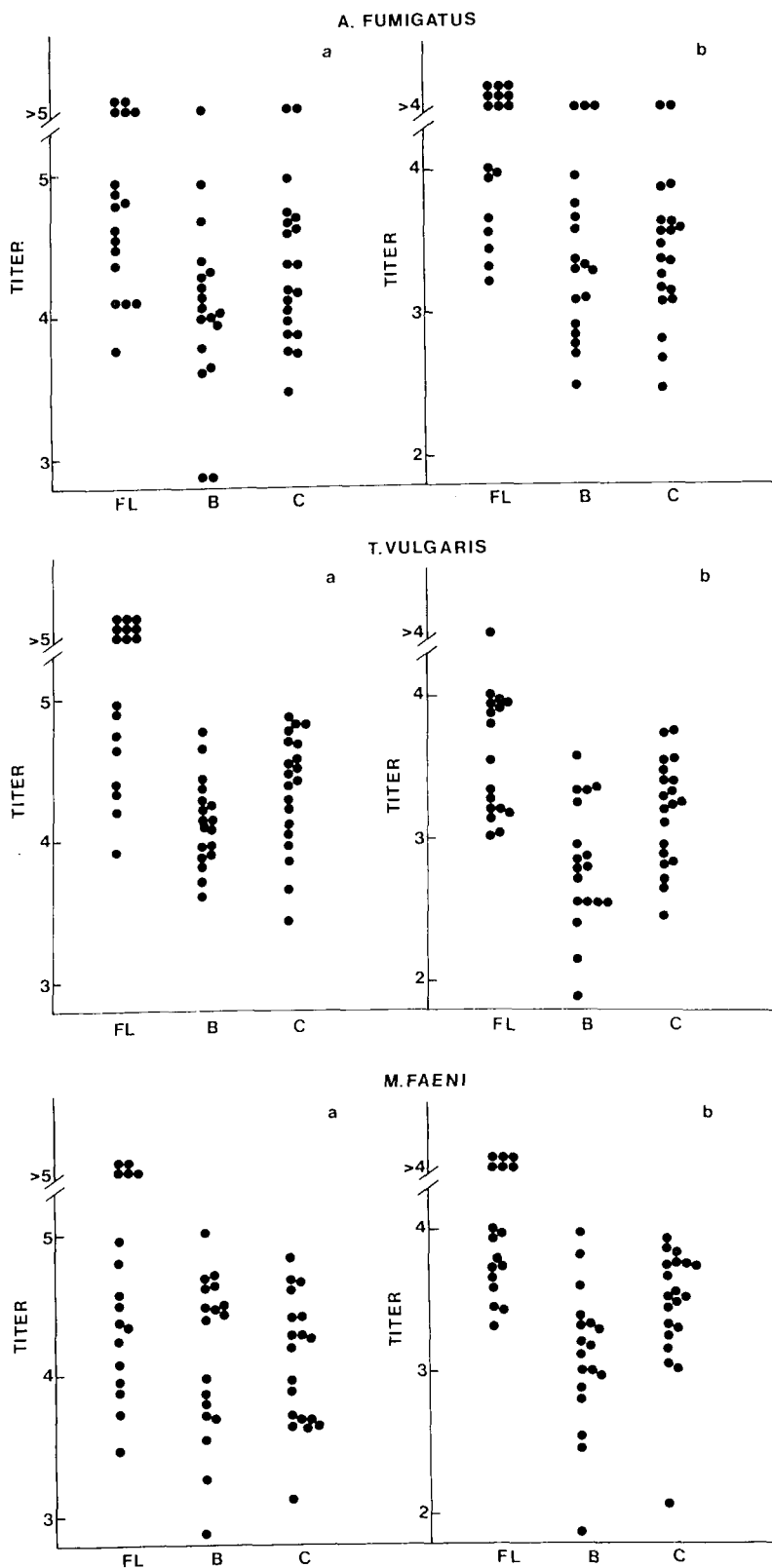


Fig. 1. Distribution of serum titers against *A. fumigatus*, *T. vulgaris* and *M. faeni* in the three test groups. Each point represents one test person. The titer is expressed as the negative logarithm of the serum dilution giving an absorbance of 0.5. FL=Farmer's lung patients, B=patients with bronchitis, C=control persons. (a) panels: mycelial antigens, (b) panels: culture fluid antigens.

and negative reactions as described in Methods. When the reactivity of individual sera with mycelial and culture fluid antigens was analyzed, some sera were found to react positively with only one of the two types of antigens (Table 1). Mycelial antigen of *A. fumigatus* gave more positive results than the corresponding culture fluid antigen. The situation was reversed for the actinomycetes, but the sera reacting only with the culture

fluid antigens were preferentially in the bronchitis and in the control group.

When the numbers of positive reactions were used to calculate antibody prevalences, the same phenomenon was also evident (Table 2). More of the serum samples had antibodies against culture fluid antigen than against the corresponding mycelial antigen of the actinomycetes. The titers of these additional positive sera were found to be just

Table 1
Comparison of serum reactivity against mycelial and culture fluid antigens

Antigen	Group							
	Farmer's lung <i>n</i> = 17		Bronchitis <i>n</i> = 18		Control <i>n</i> = 20		Total <i>n</i> = 55	
	No.	%	No.	%	No.	%	No.	%
Positive for <i>A. fumigatus</i>								
mycelial antigen only	2	12	1	6	7	35	10	32
culture fluid antigen only	1	6	2	11	2	10	5	16
Positive for <i>T. vulgaris</i>								
mycelial antigen only	3	18	0	0	0	0	3	17
culture fluid antigen only	2	12	1	6	5	25	8	44
Positive for <i>M. faeni</i>								
mycelial antigen only	2	12	1	6	0	0	3	13
culture fluid antigen only	7	41	2	11	5	25	14	58

Table 2
Prevalence of antibodies against mycelial and culture fluid antigens

Antigen	Group							
	Farmer's lung <i>n</i> = 17		Bronchitis <i>n</i> = 18		Control <i>n</i> = 20		Total <i>n</i> = 55	
	No.	%	No.	%	No.	%	No.	%
<i>A. fumigatus</i>								
mycelial antigen	13	76	4	22	9	45	26	47
culture fluid antigen	12	71	5	28	4	20	21	38
<i>T. vulgaris</i>								
mycelial antigen	10	59	0	0	0	0	10	18
culture fluid antigen	9	53	1	6	5	25	15	27
<i>M. faeni</i>								
mycelial antigen	7	41	1	6	2	10	10	18
culture fluid antigen	12	71	2	11	7	35	21	38

Table 3
Number of sera reacting with one or more of the microbial mycelial antigens

Group	Total No.	Positive against					
		One microbe		Two microbes		Three microbes	
		No.	%	No.	%	No.	%
Farmer's lung	17	6	35	6	35	5	30
Bronchitis	18	9	50	2	11	0	0
Control	20	9	45	3	15	0	0

above the limit set for positive reactions. Antibody prevalences against all three microbes were higher in the farmer's lung group than in the other two groups. However, antibodies against *A. fumigatus* mycelial antigen were also common in the bronchitis group (22%) and in the control group (45%). Patients with bronchitis and control persons who had antibodies were usually sensitized to one or two microbes whereas farmer's lung patients commonly (30%) had antibodies to all three microbes (Table 3).

DISCUSSION

ELISA has several advantages compared with other serological techniques, but it also presents some problems. One of these is the expression of the results in an informative and quantitative form. We have converted the absorbances to titers. The negative logarithm of the serum dilution giving an absorbance of 0.5 was considered as a titer, because that absorbance is still in the linear part of the absorbance curve. The use of titers indicating the highest reactive serum dilution has the advantage of familiarity from classical serology.

Another difficulty is to set the limit between positive and negative values. In preliminary tests, our reference serum was considered negative for all our antigens, but in the present series there were a few sera which showed lower titers for some antigens than the negative reference. High antibody titers to all microbes were more common in the farmer's lung group than in the other two groups. The ranges of

titer distributions were, however, wide in all groups. Positive reactions in the healthy control group reflect the fact that sensitization does not always lead to a clinical illness (6, 7, 8). On the other hand, a patient with farmer's lung may have antibodies against one microbe only and not against the others tested. This makes a comparison of the means and distributions of the antibody titers between the study groups difficult.

A number of our sera had antibodies only against the mycelial extract or against the culture fluid antigen of the microbes. This discrepancy may reflect preferential sensitization to mycelial or to extracellular antigens. This has been suggested earlier by precipitin tests for *M. faeni* (3, 4). The differences may also be caused by the different chemical nature of mycelial and extracellular antigens, especially their attachment to the polystyrene plates used in ELISA, although both protein and polysaccharide antigens apparently attach to the plates (10). Therefore it is advisable to use either a mycelial antigen or a combination of mycelial and culture fluid antigens in ELISA in the same way as in immuno-precipitation (4).

All sera reacted with the mycelial antigens almost 10 times more actively than with the culture fluid antigens despite the equal protein concentrations of the antigens. The high background reactivity of mycelial antigens was probably due to the crude nature of the antigen preparations used. Preliminary tests indicate that the background can be decreased by fractionation of the antigens.

The prevalences of antibodies by ELISA

were generally higher than those found by immunoprecipitation in previous studies by others (2, 9, 12) and by ourselves (6). This apparently is an indication of the higher sensitivity of ELISA. The same reason may explain why 30% of farmer's lung patients had antibodies to all three microbes in ELISA whereas the corresponding percentage by gel precipitation was 15% (6). A notable difference between our ELISA and precipitin results was the higher percentage of positive reactions against *M. faeni* in ELISA. The antibody prevalence by ELISA is close to those obtained previously with the precipitation test in England and the United States (2, 9). The discrepancy between ELISA and precipitin results may be due to the different antigen preparations used in precipitation and ELISA, or simply due to the higher sensitivity of ELISA. The somewhat arbitrary way of setting the limit for a positive reaction in ELISA may also contribute to these kinds of discrepancies.

The present results and previous reports of ELISA (5, 8, 10) show that ELISA, a quantitative and sensitive method, can be adapted to detect sensitization to fungal and actinomycetal antigens and used as an auxiliary test for the diagnosis of farmer's lung.

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