

Rapid enzyme-linked immunosorbent assay (ELISA) for *Aspergillus fumigatus* antibodies

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SUMMARY A rapid enzyme-linked immunosorbent assay (ELISA) where component incubation periods were shortened to one hour, was compared with agar gel double diffusion (AGDD) and a standard ELISA procedure for detecting antibodies to *Aspergillus fumigatus* in 28 asthmatic patients with suspected allergic aspergillosis. Using two *A. fumigatus* antigens the rapid ELISA compared well with AGDD and the standard ELISA method. Eleven sera that reacted with both antigens in AGDD were all positive against antigen 1 in both forms of ELISA, but two failed to react with antigen 2 in the standard ELISA and three failed to react with this antigen in the rapid method. Thirteen AGDD-negative sera were also negative in both forms of ELISA. The rapid ELISA provides a sensitive and reproducible test for routine serological investigation of allergic aspergillosis.

Serological tests are useful in the diagnosis of allergic and mycetomal forms of aspergillosis in immunocompetent patients. The usefulness of these tests in invasive forms of aspergillosis in immunocompromised patients is less well established.

Agar gel double diffusion (AGDD) is the principal serological method used at present.¹⁻⁴ Precipitins to *Aspergillus fumigatus* can be detected in the serum of almost all patients with the mycetomal form of aspergillosis. In contrast, serum from patients with allergic aspergillosis often has to be concentrated before precipitins can be detected. Other less time-consuming methods that have been evaluated for the rapid detection of *A. fumigatus* antibodies have included counterimmunoelectrophoresis,^{5,6} passive haemagglutination,⁷ radioimmunoassay^{8,9} and indirect immunofluorescence.^{10,11}

Initial investigations have suggested that enzyme-linked immunosorbent assay (ELISA) is helpful in the serological diagnosis of different forms of aspergillosis in both immunocompromised and non-compromised patients.^{12,13} However, the incubation times used in these investigations amounted to a total test period of about 24 h. This is less than that

required for AGDD, but is still too long in certain clinical situations. Although other rapid serological methods have been devised, these often have subjective endpoints and most are less sensitive. In this investigation, we have studied the effect of shortening the component incubation periods in an indirect ELISA for IgG antibodies to *A. fumigatus* to one hour. The results are compared with those obtained with AGDD and with an established long-form ELISA.

Material and methods

SUBJECTS

Serum specimens used in this investigation were obtained from 28 asthmatic patients with suspected allergic aspergillosis. These specimens were tested for *A. fumigatus* precipitins on receipt and were then stored at -20°C until required for further tests. None of the specimens had been stored for longer than 12 months when retested.

AGAR GEL DOUBLE DIFFUSION (AGDD)

Two commercial *A. fumigatus* antigens (Bencard) were used at concentrations of 20 mg/ml. The medium for this test was prepared by dissolving Noble Agar (Difco) at 3% concentration in 0.85% saline, cooling to 60°C and then adding an equal volume of McIlvaine's citrate buffer, pH 7.0. Glass

slides were coated to a depth of 1.5 mm and a template was used to cut a pattern of peripheral antigen wells of 4 mm diameter arranged 6 mm (edge to edge) from a 12.5 mm diameter central serum well. Diffusion was carried out in a moist atmosphere for 48 h. The slides were washed in 2% saline overnight, dried, stained with 0.5% Coomassie Brilliant Blue dissolved in methanol: glacial acetic acid: distilled water (5:1:5) and then destained in solvent alone. The presence of precipitin lines was recorded.

LONG ELISA

The indirect ELISA used for the determination of *A. fumigatus* antibodies was based on the method of Voller *et al.*¹⁴ Optimal concentrations of reagents were first determined using checkerboard titrations.

In subsequent tests, each well of a 96 well polyvinyl microtitre plate (No 1-220-29, Dynatech Laboratories) was first coated with 200 μ l of a solution containing 0.4 μ g of *A. fumigatus* antigen for 3 h at 37°C. The diluent used was 0.05 M sodium carbonate buffer, pH 9.6, containing 0.02% sodium azide. The coated wells were washed three times for 3 min each with PBS-T: 0.01 M sodium phosphate buffer, pH 7.2; 0.14 M sodium chloride and 0.05% (vol/vol) Tween 20 (polyoxyethylene sorbitan monolaurate) (Sigma) dispensed from a Titertek Autodrop (Flow Laboratories).

Patient serum was diluted 1/1000 with PBS-T and duplicate 200 μ l volumes were added to antigen-coated wells and incubated for 2 h at 37°C. The plates were then washed three times for 3 min each with PBS-T. Heavy-chain specific goat antiserum to human IgG labelled with alkaline phosphatase (Miles Laboratories) was diluted 1/2000 in PBS-T and 200 μ l was added to each well. The plates were incubated for 18 h at 4°C, washed three times for 3 min each with PBS-T and 200 μ l of fresh *p*-nitrophenyl phosphate (1 mg/ml), (Sigma 104 phosphatase substrate tablets) in diethanolamine buffer (diethanolamine, 97 ml; distilled water, 800 ml; sodium azide, 0.2 g; pH adjusted to 9.8 with N HCl and made up to 1 l with distilled water) was added. After incubation at 37°C for about 30 min, the reaction was stopped by adding 50 μ l of 3 M sodium hydroxide to each well.

Absorbance was read at 405 nm and 650 nm with a dual wave length MR 580 Microelisa Autoreader (Dynatech Laboratories).

RAPID ELISA

The method used was similar to the long ELISA for detection of *A. fumigatus* antibodies. Wells of polyvinyl microtitre plates were coated with 200 μ l of antigen solution consisting of 0.4 μ g diluted in

0.05 M sodium carbonate buffer, prewarmed to 37°C. The wells were coated for 1 h at 37°C in a gyratory incubator at 100 rpm. The coated wells were washed three times for 3 min each with PBS-T.

Duplicate 200 μ l amounts of patients' serum diluted with PBS-T, were added to the coated wells and incubated at 100 rpm for 1 h at 37°C. The plates were washed with PBS-T as before. Anti-human IgG labelled with alkaline phosphatase was diluted 1/2000 with PBS-T and 200 μ l added to each well and incubated at 100 rpm for 1 h at 37°C. Substrate solution was added to the washed wells and the plate incubated at 100 rpm for about 30 min at 37°C. The reaction was then stopped and read as before.

EXPRESSION OF ELISA DATA

The results were expressed as ELISA units—that is, as percentage absorbance of a reference serum dilution studied on the same occasion.¹⁵ This serum produced numerous precipitin lines in AGDD and had been obtained from a patient with the mycetomal form of aspergillosis.

Results

DEVELOPMENT OF THE RAPID ELISA FOR *A. FUMIGATUS* ANTIBODIES

The optimal concentrations of the two *A. fumigatus* antigens used in the rapid ELISA was determined by checkerboard titrations of nine AGDD-positive and nine AGDD-negative sera. A concentration of 0.4 μ g per 200 μ l of both antigens was found to be optimal.

To determine the optimal dilution of AGDD-positive and -negative sera for the rapid ELISA, serial dilutions were prepared in PBS-T and tests for the presence of IgG antibodies against *A. fumigatus* performed. The titration curves obtained with the two *A. fumigatus* antigens are presented in Figs. 1 and 2. It can be seen that good discrimination between the AGDD-positive reference serum and an AGDD-negative serum was obtained at a dilution of 1/1000 for both antigens.

INTERPRETATION OF ELISA DATA

In both forms of ELISA, discrimination between positive and negative sera was achieved by including the positive reference serum and 10 AGDD-negative sera (which had given minimal absorbance in ELISA determinations) in each test run. The mean absorbance of the 10 negative sera was determined (in ELISA units). This value plus two standard deviations was then taken as the lower limit of positiveness for that test run. Thus, patient sera were regarded as positive if the absorbance was greater than the mean plus two standard deviations of the 10 negative sera tested on the same occasion.

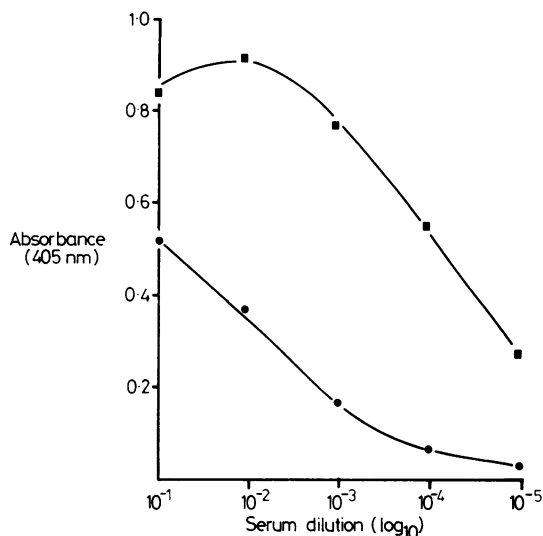


Fig. 1 Curves showing the absorbance values given by the AGDD-positive reference serum (■—■) and an AGDD-negative serum (●—●) when titrated for IgG against *A fumigatus* antigen 1

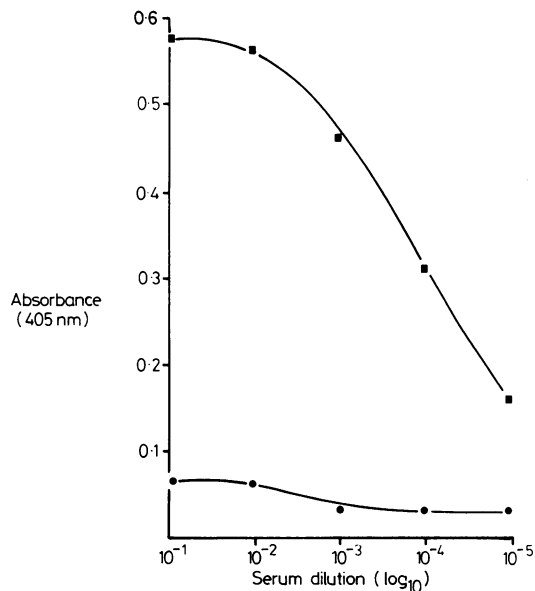


Fig. 2 Curves showing the absorbance values given by the AGDD-positive reference serum (■—■) and an AGDD-negative serum (●—●) when titrated for IgG against *A fumigatus* antigen 2

COMPARISON OF ELISA AND AGDD

The Table summarises the results obtained with the 28 sera tested for the presence of IgG antibodies against *A fumigatus* using AGDD and both forms of ELISA. Thirteen AGDD-negative sera were also negative in both forms of ELISA. Eleven sera that reacted with both *A fumigatus* antigens in AGDD all reacted with antigen 1 in both forms of ELISA, but two failed to react with antigen 2 in the long-form ELISA and three failed to react with this antigen in the rapid ELISA.

One of the two sera that reacted with antigen 1 in AGDD, but not with antigen 2 was positive in the long-form ELISA using antigen 1. Both these sera were negative in the rapid ELISA with both antigens. In contrast, two sera reacted with antigen 2 in AGDD but not with antigen 1, were positive in both

forms of ELISA with antigen 1. One of these sera was negative in the rapid ELISA with antigen 2.

Discussion

The findings of this investigation demonstrate that an ELISA method in which incubation times for each stage are reduced to one hour can be used for the rapid detection of IgG antibodies to *A fumigatus*, with no loss of sensitivity. The method compares well with the established AGDD method for the detection of *A fumigatus* precipitins.

No positive results were obtained in either form of ELISA with either *A fumigatus* antigen in tests with AGDD-negative sera. Negative ELISA results

Correlation between AGDD, long and rapid ELISA for IgG antibodies to *A fumigatus*

AGDD reaction	No of sera	Number and (%) of sera giving positive reactions			
		Antigen 1		Antigen 2	
		Long ELISA	Rapid ELISA	Long ELISA	Rapid ELISA
Negative: antigens 1 and 2	13	0	0	0	0
Positive: antigen 1, negative: antigen 2	2	1(50)	0	0	0
Negative: antigen 1, positive: antigen 2	2	2(100)	2(100)	2(100)	1(50)
Positive: antigens 1 and 2	11	11(100)	11(100)	9(82)	8(73)

were, however, obtained with antigen 2 in both forms of test with a small number of sera that had reacted with this antigen in AGDD. This problem appears to be due to poor binding of antigen 2 to the polyvinyl plates used in our ELISA tests.

Much smaller amounts of antigen and much higher dilutions of antiserum are needed for ELISA than for the established AGDD method for *A. fumigatus* precipitins. Moreover, serum specimens from patients with allergic aspergillosis often have to be concentrated before precipitins can be detected. The concentration of antigen needed for detection of precipitins in AGDD is another critical factor which can be avoided with ELISA.

The method of discrimination between positive and negative sera is one of the most critical aspects of ELISA test standardisation. In this investigation qualitative rather than quantitative results were required—that is, whether a given specimen was positive or negative. The incorporation of a positive reference serum and 10 negative sera as internal standards in each test run permitted good discrimination between negative and positive specimens and also avoided the need for critical standardisation of reaction times. This approach also lessened the need for absolute standardisation of other factors influencing reaction rates.

In conclusion, the rapid ELISA described in this paper is both sensitive and reproducible. It is simple to perform and appears to be a suitable method for routine application in the serological diagnosis of different forms of aspergillosis.

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