Comparison of immunodiffusion and enzyme linked immunosorbent assay for antibodies to four *Aspergillus* species

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SUMMARY Antigenic extracts were prepared from *Aspergillus fumigatus*, *A niger*, *A flavus* and *A terreus* for use in enzyme linked immunosorbent assay (ELISA) and immunodiffusion (ID) tests for *Aspergillus* antibodies to determine whether the use of antigenic extracts from species other than *A fumigatus* increased the sensitivity of the ELISA. ELISA titres correlated well with positive ID tests. Patient titres by ELISA were significantly higher than control titres for all species. Patient titres to *A niger* were also significantly higher than titres to the other species. Total number of ID bands to *A fumigatus* correlated significantly with anti-*A fumigatus* ELISA titres. It is concluded that the use of antigenic extracts from species other than *A fumigatus* improves the sensitivity of the ELISA.

Members of the genus *Aspergillus* may be implicated in various clinical diseases and this has been well described in previous studies.^{1,2} Serodiagnostic methods for detection of antibody have become increasingly important in the diagnosis of aspergillosis, and the most widely used of these is the immunodiffusion (ID) test, due to its simplicity and reproducibility. Its major disadvantages are lack of sensitivity and the length of time necessary to obtain a result. Several researchers have suggested that an enzyme linked immunosorbent assay (ELISA) should be used instead because of its sensitivity, rapidity, and direct measurement of antibody without reliance on "secondary phenomena."³⁻⁶

It has been shown that detection of antibody to *Aspergillus* is improved by the use of antigenic extracts from a battery of *Aspergillus* species.⁷⁻⁹ All sera received for *Aspergillus* serology by the medical mycology branch of the Western Australian State Health Laboratory Services are routinely tested by ID against extracts from four species: *A fumigatus* (two strains), *A niger*, *A flavus* and *A terreus*. *A fumigatus* is the species responsible for most cases of *Aspergillus*-related disease, and at the time of writing all reports found dealing with ELISA assays for antibody to *Aspergillus* in man dealt only with this species. This study compared ELISA and ID assays for antibody to four *Aspergillus* species to determine whether the use of antigenic extracts from species other than *A fumigatus* increased the sensitivity of the ELISA. Sera from patients with known or suspected *Aspergillus*-

related disease caused by different *Aspergillus* species were compared with those of normal controls.

Material and methods

Strains of *A fumigatus* NCPF 2140, NCPF 2109, *A niger* NCPF 2022, *A flavus* NCPF 2008, and *A terreus* NCPF 2026 were used. Antigens were prepared by mechanical disruption of mycelial mats. Cultures were grown for seven days at room temperature in 250 ml of 2% glucose, 1% peptone broth with 5% chloramphenicol (GPB) on a magnetic stirrer. These cultures were then inoculated into a further three litres of GPB and incubated for another seven days, then harvested on to gauze, washed with distilled water, dried, weighed, and stored at -20°C . A 50% w/v suspension of the mycelium was made in distilled water and disrupted for two minutes in a Waring Blender (Model 3 1 BL 44, Dynamics Corporation of America, New Hartford, Connecticut, USA). Thirty ml aliquots of the macerated material were further disrupted for four minutes in a Braun MSK Cell Homogeniser (Model 853030, Braun Instruments, San Francisco California, USA) while cooled by liquid carbon dioxide. The resulting homogenate was centrifuged at $10000 \times g$ for 10 minutes to remove gross debris. The supernate was frozen overnight, thawed, and centrifuged at $30000 \times g$ for one hour at 5°C . The supernate was dialysed against distilled water overnight at 5°C , then against polyethylene glycol 2000 at 5°C to about 20% of the initial volume. It was then frozen overnight, thawed, and centrifuged at $30000 \times g$ for one hour at 5°C . The resulting supernate was freeze-dried under

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vacuum in small aliquots and reconstituted as required to 20 mg dry weight/ml of phosphate buffered saline, pH 7.0, for use in ID tests. The *A fumigatus* ID antigen was a composite of extracts from the two strains, in equal mixture by weight.

CONTROL ANTISERA FOR ID TESTS

Control antisera were raised in New Zealand white rabbits using a regimen of subcutaneous inoculations of antigen in Freund's incomplete adjuvant and intravenous inoculations of saline preparations. Inoculum strength was 30 mg/ml. Rabbit sera were tested by ID against homologous antigens at three week intervals to assess success of immunisation.

PATIENT SERA

ID positive sera from 17 patients with different clinical forms of aspergillosis were tested by ELISA (table 1). Where possible, microscopical examination and culture of sputum was performed to check for evidence of aspergillosis. All sera were stored at -20°C until

tested by ELISA. Sex ratio was 10 men to seven women.

CONTROL SERA

Nineteen control sera were selected from patients with no clinical evidence of respiratory disease and age and sex matched as closely as possible to the patient sera.

IMMUNODIFFUSION

Agarose gel slides for ID were prepared and the slides washed, dried, and stained essentially by the method of Philpot et al,¹⁰ with minor modifications. A citrate buffer was used to eliminate possible false positive results due to C-substance. Well sizes used were: 11 mm for patient serum; 5 mm and 3 mm for antigen; and 6 mm for control rabbit antiserum. Interwell distance was 6 mm. ID was allowed to proceed for 72 hours with the gels stood in Borax-saline overnight, were dried, and stained by Coomassie blue. The relatively large patient well was used to minimise the chance of excess antigen. The smaller of the two

Table 1 ELISA and ID results from 17 patient sera

Case No	<i>A fumigatus</i>		<i>A niger</i>		<i>A flavus</i>		<i>A terreus</i>		Sputum microscopy	Sputum culture	History/diagnosis
	ID	ELISA	ID	ELISA	ID	ELISA	ID	ELISA			
1	3P	1/10240	2I	1/20480	3I	1/10240	3I	1/20480	+	<i>A terreus</i>	Allergic bronchopulmonary aspergillosis (ABPA)
2	5I	1/5120	1N	1/20480	3P	1/10240	4P	1/10240	+	<i>A fumigatus</i> <i>A niger</i> <i>A flavus</i> <i>A terreus</i>	ABPA
3	3I	1/10240	1I	1/10240	3P	1/5120	3P	1/5120	-	Non-sporing fungus	ABPA
4	6I	1/20480	2I	1/20480	1I 1N	1/20480	1P	1/10240	+	<i>A fumigatus</i> , <i>A niger</i> , <i>A terreus</i>	Long standing ABPA
5	2I	1/1280	-	1/5120	3I	1/10240	ND	ND	+	<i>A fumigatus</i>	ABPA
6	7P	1/20480	-	1/640	-	1/640	-	1/1280	+	<i>A fumigatus</i>	Chest infection, allergic rhinitis, eosinophilia, ? ABPA
7	1I	1/5120	1I	1/40960	1P	1/10240	1I	1/5120	+	<i>A terreus</i>	Asthma, on steroids, ? ABPA
8	-	<1/80	1I	1/20480	1I 1N	1/10240	ND	ND	-	<i>Aspergillus</i> species	Hayfever, atypical shadows ? ABPA
9	1I	1/640	1I	1/20480	2I	1/10240	ND	ND	+	<i>A fumigatus</i> <i>A niger</i>	Eosinophilia, ? ABPA
10	4I	1/20480	2P	1/40960	3P	1/20480	1P	1/10240	+	<i>A fumigatus</i> <i>A niger</i>	Asthma many years, ? ABPA
11	2I	1/40960	1I 1N	1/20480	1I 1N	1/20480	ND	ND	-	<i>A niger</i>	Aspergilloma and ABPA
12	2I	1/5120	2I	1/20480	2I 1N	1/10240	1I	1/2560	+	<i>A terreus</i>	Aspergilloma
13	-	1/5120	1I 1N	1/40960	1I 1N	1/10240	ND	ND	+	<i>A niger</i>	Aspergilloma
14	-	1/1280	2I	1/20480	-	1/10240	ND	ND	+	<i>A niger</i>	Aspergilloma
15	-	1/320	-	1/5120	1I 1N	1/2560	ND	ND	NA	NA	? aspergilloma
16	-	1/160	1I	1/5120	-	1/640	-	1/160	NA	NA	Cavity right lung ? aspergilloma
17	1I	1/5120	-	1/10240	1I 1N	1/10240	1I	1/2560	-	<i>A terreus</i>	Pulmonary aspergillosis two years ago, patches on chest x ray

Sputum microscopy recorded as positive if fungal mycelium present. Bands of: I = identity; P = partial identity; N = non-identity

antigen wells was added to further increase this well size differential, and to maximise detection of low patient antibody titres.

PLATES FOR ELISA

Polystyrene microtitration plates (Kayline TV 96 Plymton, South Australia) were immersed in alcohol for 15 minutes, washed for 15 minutes in detergent (Haemo-sol, Baltimore, Maryland, USA), rinsed well in deionised water, and dried. The plates were coated with 100 µl/well of appropriate antigen diluted to 0.25 mg/ml in 0.05 M carbonate buffer, pH 9.6, by incubation at 37°C for two hours, then at 4°C for about 18 hours. The antigen concentrations were pre-determined by checkerboard titration against known positive and negative human precipitating sera.

CONJUGATE

Rabbit antiserum to human IgG was purified by affinity chromatography. It was then coupled to alkaline phosphatase (Sigma 104-105, Missouri, USA) in PBS, pH 7.3, at an alkaline phosphatase protein ratio of 3:1 with 0.2% glutaraldehyde (Ladd Research Industries, Burlington Vermont, USA) which had been previously treated with solid barium carbonate to reduce acidity. After conjugation had proceeded for three hours at room temperature alkaline phosphatase antibody complexes were separated by fractionation on a column of Sephadex G200 (Pharmacia, Uppsala, Sweden) in 0.2 M TRIS-HCl buffer, pH 8.0, and stored at 4°C with 2% fetal calf serum, and 0.08% sodium azide was added.

ELISA METHOD

Plates coated with antigen were given two five minute

washes with 0.05% Tween 20 in 0.9% sodium chloride, and two-fold dilutions (1/10 – 1/20480) of patient and control sera in 100 µl volumes were made in the plates in Tween 20-sodium chloride with 20% fetal calf serum added (TW-FCS). Sera which did not reach an end-point in this titration series were titrated further. Plates were then incubated for two hours at 37°C, washed twice in Tween 20-sodium chloride, and 100 µl of conjugate diluted in TW-FCS at a pre-determined concentration (1/160) was added to each well.

After two hours of incubation at 37°C plates were again washed twice in Tween 20-sodium chloride and 100 µl of substrate (1 mg p-nitrophenyl phosphate, disodium (Sigma Chemical Co St Louis, Missouri, USA)/one ml 1M diethanolamine buffer pH 9.7 with 0.5 mM MgCl₂) added to each well. After 20 minutes of incubation at 37°C, 50 µl of 2M NaOH/well was added to stop the reaction and absorbance of the reaction product determined in a spectrophotometer (Beckman DG-B, Fullerton, California, USA) at 405 nm. Cut-off values were determined from results of a known positive and negative serum included in each batch of tests. Titres of less than 1/80 were not scored.

Results

TEST REPRODUCIBILITY

Both ID and ELISA tests showed a high degree of reproducibility. The ranking of sera by ELISA was very consistent, while actual titres were either steady or drifted by no more than one two-fold dilution. Control sera were included in each run for comparison with patient sera and with previous results.

Table 2 ELISA and ID results from 19 age and sex matched control sera

Control	<i>A. fumigatus</i>		<i>A. niger</i>		<i>A. flavus</i>		<i>A. terreus</i>	
	ID	ELISA	ID	ELISA	ID	ELISA	ID	ELISA
1	—	1/160	—	1/160	—	1/80	—	<1/80
2	—	1/640	—	1/160	—	1/160	—	1/320
3	—	1/160	—	1/160	—	<1/80	—	1/80
4	—	1/320	—	<1/80	—	1/160	—	1/80
5	—	1/160	—	1/320	—	1/160	—	1/640
6	—	1/320	—	1/1280	—	1/160	—	1/160
7	—	1/160	—	1/1280	—	1/640	—	1/160
8	—	1/640	—	1/5120	—	1/2560	—	1/1280
9	—	1/80	—	1/320	—	1/320	—	1/160
10	—	1/80	—	1/160	—	1/160	—	<1/80
11	—	<1/80	—	1/320	—	1/160	—	<1/80
12	—	1/80	—	1/1280	—	1/640	—	>1/80
13	—	<1/80	—	1/320	—	1/80	—	ND
14	—	1/320	—	1/1280	—	1/320	—	ND
15	—	<1/80	—	1/320	—	1/640	—	ND
16	—	<1/80	—	1/640	—	1/320	—	ND
17	—	<1/80	—	1/2560	trace	1/2560	—	ND
18	—	<1/80	—	1/80	—	1/160	—	ND
19	—	<1/80	—	1/2560	Ibid	1/2560	—	ND

Ibid = one band of identity.

Table 3 Overall mean ELISA titres

	<i>A fumigatus</i>	<i>A niger</i>	<i>A flavus</i>	<i>A terreus</i>
Patients	1/8954	1/19012	1/10164	1/6800
Controls	1/193	1/968	1/627	1/267
Ratio patient to control mean titres	46/1	19/1	16/1	26/1

Note: titres of <1/80 given value of 1/40 for purposes of calculation.

ELISA TITRES AND ID RESULTS FROM PATIENTS
Patient ELISA titres ranged from <1/80 to 1/40960 (table 1). Precipitin bands produced by patient sera were characterised as either bands of identity, partial identity, or non-identity with control antisera.¹¹ It was only possible to test 10 of the 17 patient sera with

A terreus antigen. Mean patient *A niger* titres were significantly higher than patient titres to the other species (Wilcoxon test; $p < 0.05$).

ELISA TITRES AND ID RESULTS FROM CONTROLS
Control ELISA titres ranged from 1/80 to 1/5120 (table 2). Two control sera with *A flavus* titres of 1/2560 were ID positive for *A flavus*, while all other control sera were ID negative. Mean control *A niger* and *A flavus* titres were significantly higher than mean control titres to the other two species (Wilcoxon test; $p < 0.05$).

COMPARISON OF PATIENT AND CONTROL ELISA TITRES
No sex differences were detected in either patient or

Fig 1 Number of patient immunodiffusion (ID) bands produced to each species compared with the natural logarithm of the reciprocal patient ELISA titre to each species. Number of ID bands to *A fumigatus* correlated significantly with anti-*fumigatus* ELISA titres (top left). No correlation between ID bands and ELISA titres for *A niger* (top right), *A flavus* (bottom left), and *A terreus* (bottom right).

control titres (Student's *t* test; $p > 0.5$). Results for men and women were therefore combined for both patients and controls (table 3). Mean patient ELISA titres were significantly higher than control titres for all species (Wilcoxon test, $p < 0.01$). There were no significant differences between the respective ratios of patient titre to control titre for all species (Student's *t* test; $p > 0.05$).

PATIENT ELISA TITRES WITH NEGATIVE ID

When ID was negative, patient ELISA titres ranged from $<1/80$ to $1/10240$. In all cases when ID was negative for a particular species, there was a heterologous positive ID test for at least one other species.

PATIENT ELISA TITRES WITH POSITIVE ID

In all instances where ID was positive, ELISA titres were $1/640$ or higher. The nature of the bands produced (whether bands of identity, partial identity,

or non-identity) showed no obvious correlation with ELISA titres for any species. Fig 1 compares the number of ID bands produced by patient sera with the natural logarithm of the reciprocal ELISA titre to the same antigen. The number of ID bands to *A fumigatus* correlated significantly with the anti-fumigatus ELISA titre. There was no correlation between number of ID bands and ELISA titres for *A niger*, *A flavus*, and *A terreus*, respectively.

ELISA TITRE DISTRIBUTION

ELISA titres were plotted for:

- (1) all patients; (2) patients with negative ID results; (3) patients with an *Aspergillus* species cultured from sputum; (4) patients with positive ID results; (5) patients with positive ID plus positive culture for *Aspergillus*; (6) controls. These results are summarised in figs 2 and 3.

In only one case where an *Aspergillus* species was cultured was the anti-fumigatus titre lower than $1/640$

Fig 2 ELISA titre distributions for *A fumigatus* and *A niger*. ● = patients; ○ = controls; ID + ve = ID positive; ID - ve = ID negative.

Fig 3 ELISA titre distributions for *A flavus* and *A terreus*. ● = patients; ○ = controls; ID + ve = ID positive; ID - ve = ID negative.

(top, fig 2). This was case 8, also ID negative for *A fumigatus*. This patient was ID positive for *A niger* and *A flavus* with correspondingly high ELISA titres. The *Aspergillus* species cultured was not one of the four species specifically tested for. No patients who were both ID positive and *Aspergillus* culture positive had an anti-*fumigatus* titre lower than 1/640. The lowest anti-*A niger* titre with an *Aspergillus* cultured from sputum was 1/640 (bottom, fig 2). This was case 6 from whom *A fumigatus* was grown from the sputum and who had an anti-*A fumigatus* titre of 1/20480. No patients who were both ID and *Aspergillus* culture positive had an anti-*A niger* titre lower than 1/20480. The lowest anti-*A flavus* titre with an *Aspergillus* species cultured from sputum was 1/640 (top, fig 3) (case 6). No patients who were both ID positive and *Aspergillus* culture positive had anti-*A flavus* titres lower than 1/10240. The lowest anti-*A terreus* titre with an *Aspergillus* cultured was 1/1280 (bottom, figure 3). This was also case six. No patients who were both ID positive and *Aspergillus* culture positive) had anti-*A terreus* titres lower than 1/2560.

ASSOCIATION BETWEEN ID AND SPECIES CULTURED

In all cases where *A fumigatus*, *A niger* and *A terreus* alone was cultured ID was positive for that particular species (none of the patients grew *A flavus* alone). The type of ID band produced bore no relation to species cultured.

ASSOCIATION BETWEEN ELISA AND SPECIES CULTURED

Mean patient titres for each species (where that species was cultured from sputum) were: *A fumigatus* 1/11413; *A niger* 1/26331; and *A terreus* 1/8530; *A flavus* was cultured only once with a titre of 1/10240. The mean patient *A niger* titre (when *A niger* was cultured) was significantly higher by the Wilcoxon test than the overall mean *A niger* titre ($p < 0.05$), and was also significantly higher than the mean patient titres to the other species when cultured ($p < 0.05$).

Discussion

Overall, positive precipitin results correlated well with ELISA titres in our study. The correlation obtained between number of ID bands and ELISA titres for *A fumigatus* agrees with the findings of Sepulveda *et al*⁵ and Kauffman *et al*,⁶ who found a definite correlation between number of ID bands to *A fumigatus* and reactivity in ELISA. Why there was no such correlation for the other three species remains uncertain, particularly as both ID and ELISA responses to these species were generally heightened when these species were cultured. These latter results may have been

statistical aberrations due to the relatively small number of samples tested—for example, *A flavus* was isolated from only one of the patients tested. The ratio of mean patient titre to mean control titre seemed to be higher for *A fumigatus* and *A terreus* than for the other two species, but this was not supported statistically due to the spread of the data.

ELISA titres were consistently high when *Aspergillus* was isolated from sputum. The cut-off point for likely ELISA titre significance (using a criterion of two titres higher than the highest respective control titre) varied among the different species, ranging from 1/2560 for *A fumigatus* to 1/20480 for *A niger*. It is important to bear in mind when making this sort of analysis, however, that a small percentage of patients may have raised antibody titres to *Aspergillus* with no evidence of disease.^{12,13}

There is some degree of serological cross reaction among different *Aspergillus* species, indicating the presence of common antigenic determinants.^{14,15} This was reflected in both our ID and ELISA results where, even when only one *Aspergillus* species was isolated from sputum, in most cases ID was positive and ELISA titres were high for several species. The possibility of false negative culture can not be discounted, however, in at least some of these cases. Cross reactions were also irregular in distribution and may reflect anamnestic recall of antibody to other previously encountered *Aspergillus* species.

Patient ELISA titres to *A niger* were significantly higher than titres to the other species, yet the rabbit-*A niger* control antiserum and patient sera tested by our laboratory generally produce fewer ID bands to *A niger* than to the other species. Control *A niger* (and control *A flavus*) titres were significantly higher than control titres for the other two species. The fact that *A niger* was the most sensitive antigen in ELISA possibly reflected different behaviour by this antigen, or various components of it, in the two systems. This might be due to a physical phenomenon such as more efficient binding to the polystyrene plates. It is interesting that De Magaldi and Mackenzie,¹⁶ when testing hyperimmune rabbit antisera by ELISA for reactivity against antigens prepared from the same *Aspergillus* strains utilised in this study, also found a relatively higher optical density with *A niger*. The reasons for this greater reactivity of *A niger* NCPF 2022 remain unknown. Further testing, using extracts from several *A niger* strains compared with several strains of each of the other species is necessary to determine whether this behaviour is species or strain specific.

Several authors have found that there is no absolute correlation between tests such as ID, counter-immunoelectrophoresis, and ELISA, and this is undoubtedly due in part to the fact that they measure

different physicochemical phenomena.^{17,18} Kauffman *et al* showed that ELISA detected antibodies against non-precipitating antigenic components, in addition to the antibodies detected by ID.¹⁹ This and a later study²⁰ also showed that precipitating antibodies in ID did not always correspond with IgG ELISA results. Further discrepancies have also been shown among IgG, IgA, and IgM ELISA titres,²⁰ and even between IgG subclass antibodies.²¹ The nature of the antigens used in all these different approaches is vitally important, and a considerable amount of work has been done on the characterisation of antigenic components of *Aspergillus* species and on their relative clinical importance.^{22,23} It would be interesting to apply some of these approaches to the use of antigenic extracts from several *Aspergillus* species in ELISA and other sensitive tests.

It was unfortunate that sera from patients with invasive aspergillosis (especially when caused by species other than *A. fumigatus*) were not available at the time of this study. A larger number of sera from patients with well defined clinical types of aspergillosis, caused by different *Aspergillus* species, needs to be examined before any firm conclusions can be drawn about ELISA titres and disease type. It was evident, however, that some patients did produce significant ELISA titres to antigenic extracts from species other than *A. fumigatus* in the absence of significant *A. fumigatus* ELISA titres. It is clear that in any investigation of immune response to *Aspergillus*, even when using sensitive tests such as ELISA, assay sensitivity may be significantly improved by using antigenic extracts prepared from a range of *Aspergillus* species.

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