The Use of an In-House Modified Double Antibody Sandwich ELISA to Detect *Aspergillus* Antigens in Sera of Immunosuppressed Patients

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ABSTRACT

Aim: **The purpose of this study was to retrospectively detect *Aspergillus* antigens in sera obtained from immunocompromised host using an in-house modified double antibody sandwich ELISA.**

Materials and Methods: **The ELISA employed the use of polyclonal antibodies raised against a water-soluble (WS) mycelial component of a clinical isolate of *Aspergillus fumigatus.* Rabbit and guinea-pig anti-WS antibodies were used as the capture and detector antibodies respectively. This was followed by the addition of anti-guinea pig antibodies conjugated to horse radish peroxidase and orthophenylenediamine.**

Results: **Of 143 specimens obtained from 140 patients, only 5 (3.5%) specimens demonstrated the presence of antigen. The 5 antigen-positive sera were from three patients. One was a confirmed case of paranasal aspergillosis and the other two were probable cases of invasive aspergillosis.**

Conclusion: **The clinical validity of the ELISA should be determined by prospective detection of *Aspergillus* antigens in multiple serum samples collected from proven cases of invasive aspergillosis.**

Keywords: invasive aspergillosis, antigen detection, ELISA

INTRODUCTION

Invasive aspergillosis is a major cause of morbidity and mortality in the immunocompromised host and particularly in those with haematological disorders[1]. The high mortality rate associated with invasive aspergillosis is attributed to the difficulties in establishing an early ante-mortem diagnosis of the disease. Ante-mortem diagnosis by sputum cultures of proven cases of invasive aspergillosis has demonstrated low sensitivities and may represent colonisation rather than infection[2]. Blood cultures of invasive aspergillosis cases are routinely negative[3] and definitive diagnosis rests on the demonstration of hyphae in histopathological sections. However, biopsies are rarely conducted as the procedure is risky in severely immunocompromised patients[2]. Since the past two decades, attention had turned to the detection of *Aspergillus* antigens in circulation as a means to earlier diagnosis. The majority of the immunoassays developed for antigen detection have been RIAs and inhibition ELISAs which have in general demonstrated moderate sensitivities but high specificities[4-6]. In comparison to the inhibition ELISAs, there have only been a few reports on the use of a double antibody sandwich ELISA for the detection of *Aspergillus* antigens[7-9]. Results of the use of these ELISAs have demonstrated higher sensitivities[8,9]. In this paper we report the use of an in-house modified double antibody sandwich ELISA for the detection of *Aspergillus* antigens in serum samples obtained from immunosuppressed patients sent to the laboratory for routine screening of fungal antigens and antibodies.

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MATERIALS AND METHODS

Fungus

The fungus used throughout the study was *Aspergillus fumigatus* M175/85, a clinical isolate from bronchial washings of a patient with a history of pulmonary abscess unresponsive to antibacterial chemotherapy.

Serum samples

A total of 143 serum samples obtained from 140 immunocompromised patients were tested for the presence of *Aspergillus* antigens. These sera were taken from patients with a clinical suspicion of fungal infection. All sera were tested retrospectively and stored at -20°C until the time of its use.

Antigen preparation

The water-soluble (WS) antigen of *A. fumigatus* M175/85 was obtained from disintegrated mycelium according to a modification of the procedure used by Hearn and Mackenzie[10]. Three-day-old starter cultures of *A. fumigatus* grown on 1% peptone and 2% glucose broth were subcultured into freshly prepared peptone-glucose broth and incubated further with agitation for 3 days at 37°C. The mycelium mats were harvested by filtration and suspended in 0.05M NH_4HCO_3 pH 8.0 and stored at -20°C for at least 24 hours until use. The mycelium rupture was

achieved by mixing the mycelium with ballotini glass beads and disintegrating the mycelium for 30 min with constant cooling (4°C) in a Dynomill (Willy A Bachofan, Baset Switzerland). The mycelium slurry was then centrifuged at 2000 g for 1 h at 4°C and the resulting supernatant which was the WS component of the mycelium was dialysed against dH_2O (Spectraphor, 14,000 dalton) and concentrated to 1/20 its original volume by dialysis against 20% (w/v) polyethylene glycol 20,000. The antigen was diluted 1:2 with glycerol, aliqouted and kept at -20°C until use.

Production of antiserum

Rabbit anti-WS antibodies was obtained by injecting subcutaneously 0.5 mL of WS antigen (0.5 mg protein concentration) mixed in an equal volume of Freund's complete adjuvant into local outbred rabbits. Thereafter 1 mL mixture of WS antigen (0.5 mg of protein) in Freund's incomplete adjuvant was injected subcutaneously every 2 weeks for a period of 12 weeks. Local outbred guinea pigs were used to raise guinea-pig anti-WS antibodies. Guinea pigs were immunised similarly to rabbits except that the WS antigen and Freund's adjuvant emulsion was given subcutaneously and intramuscularly. At the end of the immunisation period, all animals were bled and the sera obtained were pooled and stored at -20°C for future use. Before immunisation was carried out, pre-immunisation sera from rabbits and guinea pigs were collected. Serum samples demonstrating high antibody titers were pooled and purified by ammonium sulphate precipitation. The protein concentration of the purified stock rabbit and guinea pig anti-WS antibody was 10.8 and 12.6 mg mL^{-1} respectively. Each stock antiserum was diluted 1:2 in glycerol, aliquoted and stored at -20°C for future use.

A modified double antibody sandwich enzyme linked immunosorbent assay (ELISA)

Flat bottomed 96-well microtiter plates with certificate (Nunc, Maxisorb, Intermed, Denmark) was coated with a rabbit anti-WS antigen (capture antibody) diluted in 0.06M carbonate buffer, pH 9.6 for 16 h at 4°C. The optimum concentration of capture antibody required to coat plates was 1.08 μg of protein mL^{-1}. The plates were then washed thrice with 0.01M phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST), pH 7.2 – 7.4. The washings which were done in between every incubation step was carried out for 2 min using a microtiter plate shaker. Next, wells were incubated with 5% bovine serum albumin (BSA) at 37°C for 30 min in order to block non-specific binding sites. This was proceeded by the incubation with controls and test sera for 90 min at 37°C, following which detector antibody (guinea pig anti-WS antibody) at a optimum protein concentration of 1.26 μg mL^{-1} was added to the wells and incubated at 37°C for 1 hour. Next, anti-guinea pig horse radish peroxidase conjugate (Sigma, USA) at a dilution of 1:2000 was added to the wells and incubated for 1 hour at 37°C. The substrate was then added and incubated for 30 min at 37°C. The substrate consisted of 10 mg mL^{-1} of orthophenylenediamine dissolved in 0.07M citratephosphate buffer which contained 12% of H_2O_2 (4 μL of 30% (v/v) H_2O_2 in 10 mL of buffer). At the end of the incubation, positive wells imparted a yellow colour substance which turned orange upon addition of the stopping reagent ie. 2N HCL. The absorbance was read using an ELISA plate reader (Dynatech MR 5000, USA) set at a wavelength of 490 nm. The plate reader was blanked at well A1 which consisted of substrate and stopping solutions only. In every microtiter plate positive, negative, conjugate and substrate, controls were included. Positive controls consisted of 0.1 μg mL^{-1} of WS antigen diluted in 0.01M PBST containing 1% BSA (BSAPBST). Negative controls consisted of diluent or pooled normal human serum. Positive controls were incubated in quadruplicates while all other controls were incubated in duplicates. All dilutions of reagents were conducted in BSAPBST and used at a volume of 100 μL.

Standard curve

Known concentrations of WS antigens were diluted 2 fold serially in pooled normal human serum to obtain WS antigen concentrations ranging from 2 – 1000 ng mL^{-1}. These standards were included in every experiment. Concentrations of antigens in test samples were extrapolated from standard curves (WS antigen concentration versus absorbance) obtained from each test.

RESULTS

ELISA

Template absorbance value

Table I demonstrates the mean absorbance values of normal human serum in 3 separate trials ranging from 0.106 to 0.138 with an average value of 0.127. The mean standard deviation (SD) obtained from 3 trials was 0.041. The positive control (0.1 μg/mL WS antigen) was included in every trial range. The absorbances obtained for the WS antigen were 0.723, 0.616 and 0.618. The mean absorbance for the WS antigen was 0.652 . The mean values obtained from these 3 trials were referred to as the template values and all absorbance values obtained in other tests were adjusted to the template absorbance values. Adjustments were made so that results obtained in tests could be compared. The adjustments were made by multiplying a ratio to all absorbance values obtained in tests. The ratio was calculated as follows:

$$\frac{\text{Absorbance of WS antigen (0.1 }\mu\text{g/mL) in the template}}{\text{Absorbance of WS antigen (0.1 }\mu\text{g/mL) in each assay}}$$

Determination of cut-off value

The absorbance of 54 normal human serum demonstrated a log normal distribution in 3 separate trials (data not shown). Thus the cut-off value was taken as the mean absorbance value of the negative control in every test + 2SD (0..127 + 0.082). The negative control consisted of pooled normal human

Table I – The mean absorbance value of normal human serum and WS antigen

Trial	Mean absorbance value of normal human serum[a]	SD[b]	Mean absorbance value of WS antigen[c]	SD
1	0.106	0.032	0.723	0.023
2	0.138	0.047	0.616	0.032
3	0.138	0.048	0.618	0.023
Mean	0.127	0.041	0.652	0.026

[a] Mean absorbance value of 54 normal human serum
[b] SD, Standard deviation
[c] WS antigen (positive control) was used at a concentration of 0.1 μg/mL

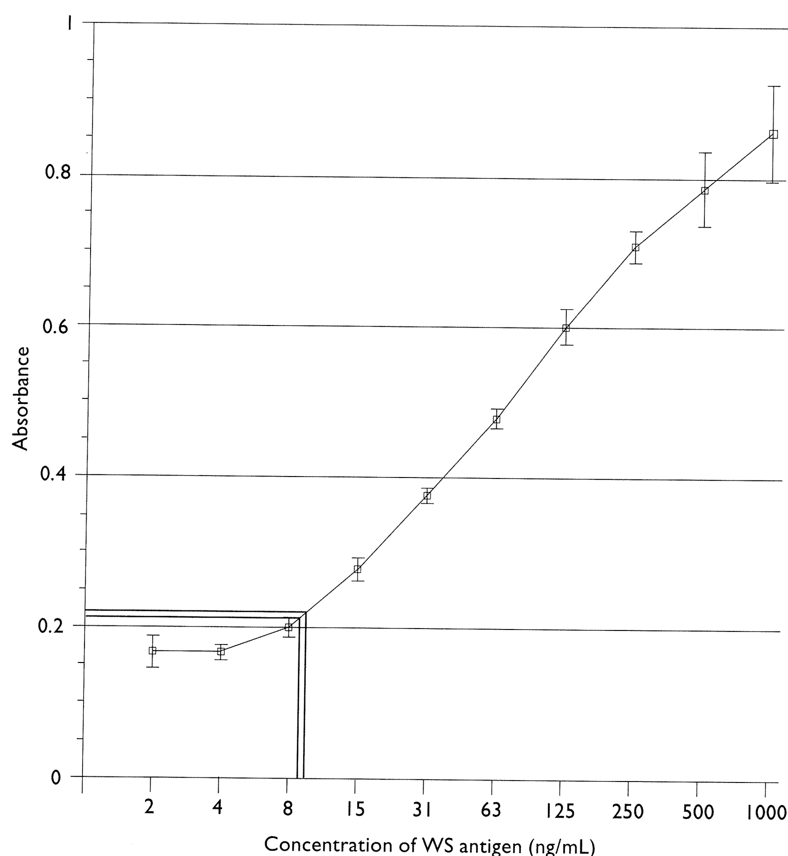

Fig 1 – Standard curve of water-soluble antigen in normal human serum.

serum which demonstrated absorbance values with differences of less than 15% of the mean absorbance value (0.127) obtained from 54 normal human sera.

Minimum detectable concentration of WS antigen

Fig 1 shows the standard curve for WS antigen concentration ranging from 2 – 1000 ng mL^{-1}. The standard curve is an average of 3 curves obtained in separate immunoassays. The minimum detectable concentration of WS antigen was extrapolated graphically from standard curves obtained from each immunoassay. The intersection of the cut-off value with the standard curve yielded the minimum detectable concentration of antigen in each assay. The cut-off absorbance value from 3 separate immunoassays were 0.210, 0.210 and 0.220. These cut-off values corresponded to minimum detectable WS antigen concentrations of 8.7, 8.7 and 9.0 ng/mL with an approximate average of 9.0 ng/mL.

Detection of *Aspergillus* in sera of immunocompromised patients

Of the 143 serum samples tested, only 5 samples from 3 patients were positive for *Aspergillus* antigen. The absorbance values for positive sera were higher (range: 0.250 – 0.280) than the absorbance values of 96% of normal human sera tested (range: 0.045 – 0.208). Case 1 was a confirmed case of paranasal aspergillosis (Table II). *Aspergillus fumigatus* was cultured from biopsy material taken from the paranasal sinuses. Three consecutive serum samples taken from this patient were positive for antigen with concentrations ranging from 12 – 15 ng/mL. Two other patients demonstrated antigen concentrations of 12 ng/mL.

DISCUSSION

The incidence of invasive aspergillosis (IA) has increased 8-fold in the past three decades[1]. In some institutions, the incidence of aspergillosis is the same as candidosis or have superceded candidal infections in leukemic patients. Invasive aspergillosis constitute as high as 55% of all fungal infections in leukemic patients[11]. The overall mortality of untreated IA is high[1] but can be reduced with prompt diagnosis and early initiation of therapy[12]. However, the major

Table II – Clinical summary and mycological investigations of patients with antigen positive serum

Patient	Sex	Age	Race	Clinical diagnosis	Culture	Antibody[a]		Antigen[b]		Aspergillus Ag[c]
						Asp[d]	Cand[e]	Cand	Crypto[f]	(μg/mL)
(1) JM	M	49	I[g]	Fungal sinusitis	+	+	nd[h]	nd	nd	0.015 0.012 0.012
(2) MS	M	5	M[i]	Acute lymphoid leukemia	nd	-	-	-	-	0.012
(3) IS	M	2	M	Lung infection	nd	-	+	nd	nd	0.012

[a] Antibody detected by counter-immunoelectrophoresis
[b] Antigen detected using commercial latex agglutination kits
[c] Antigen detected using the in-house ELISA
[d] *Aspergillus*
[e] *Candida*
[f] *Cryptococcus*
[g] Indian
[h] Not done
[i] Malay

obstacle to successful management of invasive aspergillosis in the inability, confirm the diagnosis early in the course of infection.

Conventional laboratory test of direct microscopy and culture are of limited value for the diagnosis of IA[2,3]. Due to this, an in-house ELISA was devised earlier for the detection of *Aspergillus* antigens in rabbits with invasive aspergillosis[13]. In rabbits, the ELISA demonstrated a sensitivity of 73% and a specificity of 99% for the detection of antigens in rabbit sera. The ELISA could also be completed in 5 hours following an overnight coating step. The ELISA conducted in this study was shortened to 4h 30 min by decreasing the incubation step of specimens from 120 min to 90 min as results obtained earlier for both incubation periods were comparable. Coating antibody and detector antibody was used at a final concentration of 1.08 μg/mL and 1.26 μg/mL respectively. A 1 – 10 μg of protein is normally recommended for coating microtiter plates[14]. Since the concentrations of stock solutions of capture and detector antibodies were high, a 10,000 fold dilution was necessary to achieve working dilutions. This was beneficial as only 1 μL of each antiserum was sufficient in each immunoassay. The other benefit is that high dilutions of stock antibodies may eliminate the presence of non-specific antibodies which may interfere with the test. The minimum detectable concentration (MDC) of WS antigen in human sera in this study was 9 ng/mL. In a previous study using the ELISA in a rabbit model of invasive aspergillosis, the MDC was 2.3 ng/mL. The differences in MDC values obtained for rabbits and humans can be attributed to the higher absorbance readings and greater distribution of absorbance values of normal human serum as compared to normal rabbit serum.

In this study, antigen was detected in only 5 of the 143 serum samples tested ie. an antigen detection frequency of 3.5%. The low antigen detection rate may be likely due to the following: i) antigen detection was conducted retrospectively on samples obtained from immunocompromised patients. These specimens were sent for routine detection of fungal antigens and/or antibodies; ii) most serum samples were obtained from patients with a risk of acquiring any invasive fungal infections and not necessarily invasive aspergillosis alone.

The antigen rates obtained here are the same as that reported by Wilson et al[7] when single serum samples from patients with a likelihood of acquiring invasive aspergillosis were examined for *Aspergillus* antigen retrospectively. On the other hand, when this group of researchers tested two or more serum samples per patient, the frequency of antigen detection was increased to 19.2%. Examination of multiple rather than single specimens has also been shown by Johnson et al[6] to increase antigen detection rates. In proven cases of invasive aspergillosis antigen, detection rates ranged from 67% to 95% using either radioimmunoassays or ELISAs of different designs[3,15].

The confirmed case of aspergillosis in this study was a case of fungal sinusitis with complications. A 49-year-old male patient with a history of right-sided headache presented with proptosis of the right eye and eventual blindness of the same eye. A computerised tomography (CT) scan revealed a sphenoethmoidal mass and culture of biopsy material taken from this mass grew *Aspergillus fumigatus*. In addition, of three consecutive serum samples being positive for antigen, one sample which was tested was also positive for *Aspergillus* antibody. The two other patients who demonstrated *Aspergillus* antigens in single specimens may have been cases of invasive aspergillosis as there was clinical suspicion for the infection.

The clinical validity of the in-house ELISA for antigen detection can only be determined when proper steps have been taken for antigen detection. A prospective study of multiple serum samples taken from each patient with proven invasive aspergillosis would be suitable for determining the sensitivity of the test. The specificity of the immunoassay can be obtained by testing normal healthy individuals and at risk patients who do not have the disease. However, the procurement of proven cases of aspergillosis is notoriously difficult and one would have to resort to patients with highly probable cases of IA as determined by clinical and microbiological suspicions.

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Appendix: Statements to which subjects were asked to respond to*

Please check the box for the answer that most accurately describes how often the following statements apply for you.

	Always	Usually	Many times	Once in a while	Almost never	Never
1. I feel guilty when I eat too many sweets	☐	☐	☐	☐	☐	☐
2. I think that my stomach is too big.	☐	☐	☐	☐	☐	☐
3. I eat when I am upset.	☐	☐	☐	☐	☐	☐
4. I think about dieting.	☐	☐	☐	☐	☐	☐
5. I am rather picky about what I eat.	☐	☐	☐	☐	☐	☐
6. I stuff myself with food.	☐	☐	☐	☐	☐	☐
7. Sometimes I start eating too much and cannot sleep.	☐	☐	☐	☐	☐	☐
8. I think I should be a little heavier.	☐	☐	☐	☐	☐	☐
9. I think I should be somewhat taller.	☐	☐	☐	☐	☐	☐
10. I think I am too heavy.	☐	☐	☐	☐	☐	☐
11. I think about being thinner alot.	☐	☐	☐	☐	☐	☐
12. I like my body shape and size.	☐	☐	☐	☐	☐	☐
13. I cannot eat when I am deeply worried about something.	☐	☐	☐	☐	☐	☐
14. I do not worry about my weight.	☐	☐	☐	☐	☐	☐
15. I am quite satisfied with my present weight.	☐	☐	☐	☐	☐	☐
16. I am quite satisfied with my present height.	☐	☐	☐	☐	☐	☐

* Affirmative responses to statements 2,4,8,10,11 were taken to be indicative of dissatisfaction with body size/shape. Affirmative responses to statements 12,14,15 are related to satisfaction with body size/shape.