ORIGINAL ARTICLE

The Use of an In-House Biotin-Avidin Linked Immunosorbent Assay to Detect *Aspergillus* Antigens in Sera of Immunocompromised Patients

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Summary

A biotin-avidin-linked immunosorbent assay was developed to detect *Aspergillus* antigens in sera of immunocompromised patients. The assay was based on a double antibody sandwich ELISA using polyclonal antibodies raised against water-soluble antigens of *Aspergillus fumigatus*. *Aspergillus* antigens were positive in sera of 9 of 16 (56%) patients who were studied prospectively and in 13 of 73 (19%) patients studied retrospectively. The 9 prospectively studied patients who were antigen positive were febrile neutropenic hematological malignancy patients who exhibited a high risk of acquiring invasive aspergillosis.

Key Words: Biotin-avidin linked immunosorbent assay, *Aspergillus* antigen detection, Hematological malignancy

Introduction

Invasive aspergillosis (IA) is an increasingly recognized condition in the immunocompromised host[1]. Patients particularly at risk of acquiring IA are patients with hematological malignancy during periods of prolonged and profound neutropenia[1]. The mortality rate of IA remains high i.e. about 90% even when treated[2]. The high mortality rate may partly be attributed to difficulties in obtaining definitive diagnosis at an early stage of the disease. Definitive proof of IA implies the histopathological evidence of hyphae in tissue together with the isolation of *Aspergillus* from the same specimen. However, the performance of invasive techniques to obtain biopsies is often precluded by thrombocytopenia or by the critical condition of the patient[3]. Microbiological techniques of sputum culture to isolate *Aspergillus* lack sensitivity[3] and blood cultures are virtually all negative even in proven cases[4]. Over the past two decades, attention has been focused on the detection of circulating *Aspergillus* antigens as a means of earlier diagnosis. The majority of the immunoassays like radioimmunoassays[5], enzyme linked immunosorbent assays[6] and latex particle agglutination tests[7] detect galactomannan, a heat stable cell wall component of *Aspergillus* which is released during invasive disease. Others[8,9] have detected protein antigens in serum and/or urine of humans or animals with IA using Western blot techniques. The aim of developing an immunoassay is to procure a technique which is sensitive, specific and

which demonstrates a low enough threshold for antigen detection. The antigen detected should also be an early marker of infection. The commercially available ELISA kit (Platelia *Aspergillus*, Sanofi Diagnostics Pasteur, France) which utilizes monoclonal antibodies to detect the galactomannan antigen has demonstrated high sensitivity[10] and early detection of the antigen[11]. The high sensitivity of the commercial ELISA is attributed to the sandwich ELISA technique which facilitates the detection of the galactomannan at low (ng/ml) concentrations. In our laboratory, an in-house double antibody sandwich ELISA employing polyclonal antibodies was earlier developed to detect uncharacterized *Aspergillus* antigens in experimentally infected rabbits (unpublished observations). The sensitivity and the specificity of the test were 73% and 99% respectively. This technique was later shown to be useful for the detection of circulating *Aspergillus* antigens in serum samples of a patient with proven invasive aspergillosis[12]. This paper reports the development of a biotin-avidin linked immunosorbent assay (BALISA) to detect circulating *Aspergillus* antigens in sera of immunocompromised patients. The BALISA is a modification of the original in-house ELISA which includes a biotin-avidin amplification step aimed at improving the sensitivity of the immunoassay.

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 100 serum samples obtained from 89 immunocompromised patients were tested for the presence of *Aspergillus* antigens. Eighty serum samples from 73 patients were tested retrospectively whilst 20 sera from 16 patients were tested prospectively. Sera tested retrospectively were from generally immunocompromised patients not responding to antibacterial antibiotics. Sera tested prospectively were from patients with hematological malignancies. These patients were febrile neutropenics who were not responding to broad spectrum antibiotics after 1 week of treatment. The patients were also clinically suspected of having fungal infection. All sera were 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 (1979)[13]. 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 h until use. The mycelium rupture was achieved by mixing the mycelium with ballotini glass beads and disintegrating the mycelium for 10 min with constant cooling (4-8°C) in a Dynomill (Willy A. Bachofan, Basel, Switerzland). 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, aliquoted and kept at -20°C until use.

Production of antiserum

Rabbit and guinea-pig anti-WS antibodies were obtained as described earlier[12]. At the end of the immunization period, all animals were bled and the sera obtained were pooled and stored at -20°C for future use. Before immunization was carried out pre-immunization 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 antibodies were 13.4 and 11.1 mg/ml respectively. Each stock antiserum was diluted 1:2 in glycerol, aliquoted and stored at -20°C for future use.

The Biotin-Avidin-Linked Immunosorbent Assay (BALISA)

The BALISA was a modification of an in-house double antibody sandwich ELISA described previously (Samad and Rahman, 1999). Flat bottomed 96-well microtiter plates with certificate (Nunc, Maxisorb, Intermed, Denmark) were coated with a rabbit anti-WS antibody (capture antibody) diluted in 0.06M carbonate buffer, pH 9.6, at a protein concentration of 1.34 µg/ml. Coating of the plates were conducted overnight for 16 h at 4°C. The following morning, the wells were washed thrice with 0.01M phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST), pH 7.2-7.4, before subsequent incubations were carried out. All subsequent incubations were conducted in a water-bath (Grant, UK) heated to 37°C. In between each incubation step, wells were washed thrice with PBST. Wells were first incubated with 5% bovine serum albumin (BSA) for 15 min in order to block non-specific binding sites. This was proceeded by incubating the control and test sera for 45 min. Next, detector antibody (guinea pig anti-WS antibody) at a protein concentration of 1.11 µg /ml was added to the wells and incubated for 30 min. Anti-guinea pig biotin conjugate (Sigma, USA) at a dilution of 1:20 000 was then added to the wells and incubated for 30 min. The substrate was then added and incubated for 30 min. The substrate orthophenylenediamine (OPD) which was used at a concentration of 0.01% [1mg/ml of OPD dissolved in 0.07M citrate-phosphate buffer] contained 0.012% of H_2O_2 (4µl of 30%(v/v) H_2O_2 in 10ml of buffer). At the end of the incubation, positive wells imparted a yellow colour which turned orange upon addition of the stopping reagent i.e. 2N HCL. The absorbance was read using an ELISA plate reader (Dynatech MR 5000, USA) set at a wavelength of 490nm. 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 0.13µg/ml of WS antigen diluted in pooled normal human sera or 0.01MPBST containing 1% bovine serum albumin (BSAPBST). Negative controls consisted pooled normal human serum. Positive and negative 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 100µl. Avidin peroxidase (Sigma, USA) at a dilution of 1:200,000 was next incubated for 30 min.

Standard curve

Known concentrations of WS antigens were diluted 2 fold serially in pooled normal human serum (160 healthy blood donors) to obtain WS antigen concentrations ranging from 0.002 - 1(µg/ml. 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.

Latex agglutination test

The Pastorex *Aspergillus* kit (Sanofi Diagnostics Pasteur, France) was used to detect galactomannan antigen in sera of some patients. This was conducted as part of our routine diagnostic services for sera obtained from patients with hematological malignancies. The storage of the kit and the protocol for antigen detection were strictly adhered to the manufacturer's instructions. Briefly, 300µl of patient's serum was mixed with 100µl of treatment buffer (EDTA) in an Eppendorf tube and boiled for 3 min at 100°C. The Eppendorf tube was then centrifuged and 40µl of the resulting supernatant was mixed with 10µl of the latex reagent. The card containing this mixture was agitated for at least 5 min on a horizontal rotary shaker before observation for agglutination was conducted. Agglutination seen with the naked eye was interpreted as positive provided positive and negative controls gave expected reactions. Positive and negative controls consisted of galactomannan antigen and glycine buffer respectively.

Results

BALISA

Template Absorbance Values

Table I demonstrates the mean absorbance values and standard deviations of positive control (PC), negative control (NC) and 160 individually tested normal human sera (NHS). The mean absorbance readings obtained from 160 individually tested normal human sera were 0.281, 0.261 and 0.222 in 3 separate trials. The mean of these 3 absorbance values was 0.255 and the mean

Table I
Mean absorbance value and standard deviation of WS antigen and normal human serum

Trial	PC[a]	SD	NC[b]	SD	NHS[c]	SD[d]
1	0.815	0.044	0.297	0.009	0.281	0.009
2	0.741	0.053	0.262	0.032	0.261	0.031
3	0.685	0.063	0.221	0.023	0.222	0.019
Mean	0.747	0.053	0.260	0.021	0.255	0.020

a PCS; Positive control constituted 0.13ug/ml of WS antigen prepared in pooled normal human serum.
b NC; negative control constituted pooled human serum from 160 individual normal human sera.
c NHS; 160 normal human sera which were tested individually and presented as the mean absorbance value.
d SD; standard deviation

standard deviations from the 3 trials for normal human sera was 0.020. The mean absorbance value and the standard deviation of the negative control of 0.260 and 0.021 respectively were comparable to the mean absorbance value and standard deviation of the 160 individually tested normal human sera. The mean absorbance value for the positive control was 0.747 and the mean standard deviation 0.053. The mean absorbance values obtained from these 3 trials were referred as to the template values and all absorbance readings 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 other tests. The ratio was calculated as follows:

$$\frac{\text{Absorbance of WS antigen (0.1ug/ml) in the template}}{\text{Absorbance of WS antigen (0.1ug/ml) in every assay}}$$

Determination of Cut-Off Absorbance Value

The absorbance values of the 160 normal human sera in 3 separate trials demonstrated a normal distribution (data not shown). The cut-off absorbance value was taken as the mean absorbance value of the negative control in every test + 2SD. The cut-off absorbance value from 4 trials conducted on different days were 0.285, 0.299, 0.299 and 0.298 with a mean value of 0.295 (mean:0.255 + 2SD:0.040) (Table II).

Table II
The minimum detectable concentration of WS antigen as obtained by interpretation and from extrapolation of standard curve of the in-house BALISA

Trial	Cut-off Absorbance Value X + 2SD)	Interpretation µg/ml	Extrapolation from curve µg/ml
1	0.285	0.002	0.001
2	0.299	0.004	0.003
3	0.299	0.004	0.003
4	0.298	0.004	0.004
Mean	0.295	0.004	0.003

Minimum Detectable Concentration of WS Antigen

The minimum detectable concentration (MDC) was determined as the lowest concentration of WS antigen that demonstrates an absorbance value greater or equal to the cut-off absorbance value determined in each assay. The MDC was determined either by the above interpretation or by extrapolation of the value from the standard curve of 2 fold serially diluted WS antigens ranging from 0.002ug/ml to 1ug/ml. The MDC based on interpretation for separate trials were 0.002ug/ml, 0.004ug/ml, 0.004ug/ml and 0.004ug/ml respectively with a mean MDC of 0.004ug/ml (Table II). The MDC values obtained from extrapolation of the normal curve for 4 separate assays were 0.001ug/ml, 0.003ug/ml, 0.003ug/ml and 0.004ug/ml respectively with a mean value of 0.003ug/ml (Table II and Figure 1).

Detection of *Aspergillus* Antigens in Sera of Immunocompromised Patients

Of the 100 sera tested retrospectively and prospectively from 89 patients, 26 sera were positive for *Aspergillus* antigen. *Aspergillus* antigens were detected more frequently in sera of patients who were prospectively studied [56% (9 of 16 patients)] than in retrospectively studied patients [18% (13 of 73 patients)] (Figure 2).

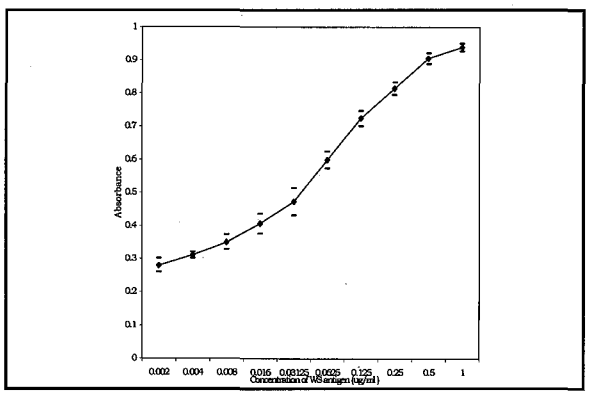

Figure 1:Standard curve of the in-house BALISA

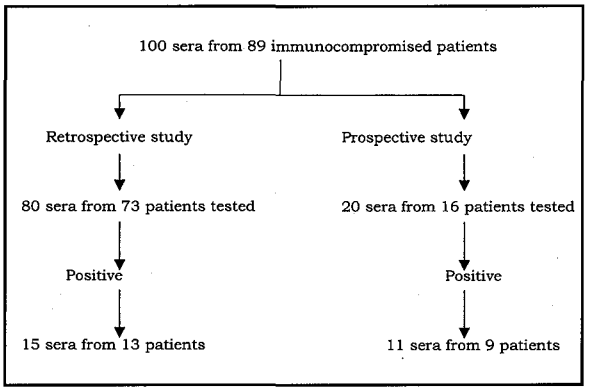

Figure 2: The number of sera from immunocompromised patients that were tested positive retrospectively and prospectively

With regards to the percentage of sera being tested positive, 15 of 80 (19%) sera from the retrospectively tested group were positive whilst 11 of 20 (55%) sera from the prospectively tested group were positive. Of the 11 sera from prospectively studied patients that were positive for *Aspergillus* antigen with the BALISA, 9 were positive with the commercial latex agglutination kit (Pastorex *Aspergillus*, Sanofi Diagnostics Pasteur, France).

Discussion

An in-house double antibody sandwich ELISA was previously developed to detect *Aspergillus* antigens in sera of rabbits with experimental invasive aspergillosis. The sensitivity and specificity of the ELISA was 73% and 99% respectively. The in-house ELISA was later tested in a retrospective study to detect *Aspergillus* antigens in sera of generally immunosuppressed patients. Although the ELISA detected *Aspergillus* antigens in 3 consecutive sera of a patient with confirmed paranasal aspergillosis, only 3.5% (5 of 143) of the tested sera were found to be positive[12].

The present study attempts to improve the sensitivity of the original in-house ELISA by using a biotin-avidin amplification system. The modification of the original in-house ELISA was the addition of anti-guinea pig biotin conjugate following incubation with the detector antibody. This additional step however, did not incur an overall increase in duration of incubation. On the other hand, because incubation was conducted in the water-bath, the incubation period was shortened to 3 hours following an overnight coating step for the capture antibody. The duration of incubation for the former in-house ELISA was 4h 30min and incubation was conducted in an incubator. For the BALISA the capture and detector antibodies were used at a protein concentration of 1.34 and 1.11ug/ml respectively. A similar concentration of capture and detector antibodies were also used in the in-house ELISA. A protein concentration of 1-10ug/ml is generally recommended for coating microtiter plates[15].

The minimum detectable concentration of WS antigen in sera for the BALISA as obtained from extrapolation of the standard curve was 0.003ug/ml. The previous in-house ELISA demonstrated a MDC of 0.009ug/ml [12]. Thus, the biotin-avidin amplification system managed to increase the sensitivity (the threshold level of antigen detection) of the ELISA by 3 fold. This was noticed as an increased level of antigen detected in sera of patients. A formerly proven case of invasive paranasal aspergillosis demonstrated antigen levels of 0.012 - 0.015ug/ml in 3 consecutive sera when tested with the ELISA, whilst the levels of antigen detected in the same serum samples of this patient using the BALISA was 0.297 - 0.300ug/ml. Fujita et. al[15] who used a biotin-streptavidin ELISA to detect *Aspergillus* antigens reported a MDC of 0.0016ug/ml for a heat stable antigen and the antigen levels in sixteen patients in their study, ranged from

Table III
Patient details and results of antigen detection by in-house BALISA and latex agglutination test

Patient No.	Age/Race/ Sex	Underlying disease	Sera tested by:		Concentration of WS antigen (ug/ml)[a]
			BALISA	LA	
1.	37/M /M	Acute myeloid leukemia	+	+	0.315
2.	61/M/F	Aplastic anaemia	+	+	0.327
			+	+	0.336
3.	13/M/F	Acute myeloid leukemia	+	+	0.331
4.	59/MF	Myelodysplastic syndrome	+	+	0.300
5.	64/M /F	Multiple myeloma	+	-	0.287
6.	60/C/M	Myelodysplastic syndrome	+	+	0.308
7.	37/M/M	Acute myeloid leukemia	+	+	0.304
			+	+	0.313
8.	13/M/F	Aplastic anaemia	+	+	0.286
9.	67/C/M	Myelodysplastic syndrome	+	-	0.306

a *Concentration of water-soluble (WS) antigens determined with the in-house BALISA*

0.002 - 0.017ug/ml. In this study, the antigen concentrations in 9 patients who were followed up prospectively ranged from 0.286 - 0.327ug/ml. Differences in antigen concentrations detected may be due to the different types of antigens being detected, variations in the double antibody sandwich technique and differences in patient population. In this study, a direct comparison of sensitivity (frequency of antigen detection) between the BALISA and the previous in-house ELISA cannot be made as identical serum samples were not tested. On the other hand, if a comparison of retrospectively tested sera is made, the sensitivity of the in-house ELISA was found to be at least 5 fold lower (3.5%) than the BALISA which demonstrated an antigen detection rate of 19%.

The overall antigen detection rate using the BALISA was 25% (22 of 89 patients). Antigen detection rates of sera of patients who were tested prospectively was 56% whilst the antigen detection rates of sera of patients tested retrospectively was 19%. The reason for the higher antigen detection rates in patients followed prospectively was because these patients were cases in which invasive aspergillosis were suspected. The 9 patients who were positive for *Aspergillus* antigens were patients with hematological malignancy, who were febrile and neutropenic and were not responding to broad spectrum antibiotics. All these patients demonstrated pulmonary infiltrates on X-rays. One patient (patient number 8) exhibited consolidation and cavitary lesions of the lungs upon computed tomography scan examination. The appearance of cavitary lesions is highly suggestive of invasive fungal infections[16]. Cultures of sputum and blood from 5 of these patients and urine cultures from 7 of these patients however, failed to isolate aspergilli. This is not an unexpected finding as conventional laboratory test like direct microscopy and culture are of limited value in the diagnosis of IA [3,4]. Of the 11 sera from the 9 patients which were tested positive with the BALISA, 9 sera were found to positive with the commercial latex agglutination kit. The commercial latex agglutination kit (Pastorex *Aspergillus*, Sanofi Diagnostics, France) detects the galactomannan antigen[7] whilst the antigens detected by the BALISA is speculated to predominantly be proteinaceous in nature. This speculation is based on the observation that heating the sera prior to conducting the test resulted in considerable loss of reactivity. The

galactomannan antigen on the other hand is a cell wall polysaccharide antigen which is resistant to boiling and treatment with proteases [6]. The biotin-avidin sandwich ELISA employed by Fujita et. al[15] demonstrated a sensitivity and specificity of 89% and 99% respectively in detection of heat-stable *Aspergillus* antigens for patients with proven invasive aspergillosis. In the present study, the sensitivity and specificity could not be established as cases obtained were not proven cases of invasive aspergillosis. A prospective study to identify proven cases of invasive aspergillosis and to detect antigens in multiple serum samples taken from patients with hematological malignancy is currently in progress.

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