

Detection of *Aspergillus* Antigens in Experimental Invasive Aspergillosis using a Biotin-Avidin Linked Sandwich ELISA (BALISA)

Shamim Abdul Samad^{1*}, Jacinta Santhanam², Hamidah Yusoff¹

¹Department of Medical Microbiology & Immunology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latiff, Bandar Tun Razak, Cheras, 56000 Kuala Lumpur, Malaysia

²Department of Biomedical Sciences, Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

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Abstract. A biotin-avidin amplified sandwich ELISA utilizing polyclonal antibodies to water soluble (WS) mycelial antigens of *Aspergillus fumigatus* was used to detect antigens in sera of rabbits with experimental invasive aspergillosis. Tricolor rabbits were more susceptible to infection than NZW rabbits as evidenced by a faster progression of infection and greater isolation rates of *A. fumigatus* from organ culture. Antigenemia was detected in all 11 Tricolor rabbits inoculated with 1×10^6 and 1×10^7 conidia. However, antigenemia was detected in only 0%, 50% and 75% of NZW rabbits inoculated with 1×10^6 , 1×10^7 and 1×10^8 conidia, respectively. Tricolor rabbits demonstrated antigenemia in 75% (18 of 24) of sera, whilst only 36% (5 of 14) of sera of NZW rabbits tested positive. The overall sensitivity and specificity of antigen detection in Tricolor and NZW rabbits were 84.2% (16 of 19 rabbits) and 85.7% (12 of 14) respectively. The protein rich Concanavalin A unbound fraction of WS antigens demonstrated immunoreactivity with the BALISA. Immunoblot studies using the capture antibody demonstrated 3 strongly immunoreactive regions of 38-46kD, 66-68kD and 73-85kD for WS antigens. The results of partial characterization of WS antigens, suggests that the antigens detected are protein in nature.

Keywords. Antigen detection, experimental invasive aspergillosis, sandwich ELISA

INTRODUCTION

Invasive aspergillosis is a major cause of mortality and morbidity in immunosuppressed hosts and has become the leading cause of death amongst hematology patients (Latge, 1999). The mortality remains high i.e. about 90% amongst these patients and it is partly attributed to the difficulties in diagnosing the infection at an early stage of the disease (Bodey *et al.*, 1992). Definitive proof of invasive aspergillosis requires histopathological evidence of hyphae in tissue together with the isolation of *Aspergillus* from a sterile site (Ascioglu *et al.*, 2002). However, the performance of invasive techniques to obtain biopsies is often precluded by thrombocytopenia or by the critical condition of the patient. Microbiological techniques of sputum culture to isolate *Aspergillus* lack sensitivity (Meyer *et al.*, 1973) and blood cultures are virtually negative in proven cases (Young *et al.*, 1970). Earlier diagnosis and better patient management has resulted from conducting routine high resolution CT scans on patients suspected with invasive aspergillosis (Maillot *et al.*, 1997). The presence of a halo sign of the thorax in febrile neutropenics is an early indicator and highly suggestive of invasive aspergillosis (Denning, 1998). 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 immunoassays like radioimmunoassay, enzyme linked immunosorbent assay and latex particle agglutination tests detect galactomannan, a heat stable cell wall component of *Aspergillus* which is released during invasive disease. The commercially available sandwich ELISA kit (Platelia *Aspergillus*, Sanofi Diagnostics, Pasteur) which utilizes monoclonal antibodies to detect the galactomannan has demonstrated antigen threshold detection of less than 1ng/ml and is by far the most sensitive antigen detection test available for invasive aspergillosis (Stynen *et al.*, 1995). The Platelia *Aspergillus* has demonstrated high sensitivities and early detection of the antigen prior to clinical and / or radiological suspicion of the disease (Maertens *et al.*, 1999). On the other hand, false positive results of up to 18% have been reported (Yeo and Wong, 2002) and has been attributed to galactomannan found in certain foods which may reach the circulation through damaged intestinal mucosa (Lescher-Bru *et al.*, 1998) caused by cytotoxic chemotherapy. The other drawback of detecting the galactomannan antigen is its transient presence due to its efficient removal by Kupffer

*Author for Correspondence.

Mailing address: Associate Professor Shamim Abdul Samad, Dept. Medical Microbiology & Immunology, Faculty of Medicine, UKM, Jalan Yaacob Latiff, 56000 Kuala Lumpur, Malaysia. Tel: 603-91702208; Fax: 603-91737336
Email: shamim@mail.hukm.ukm.my

cells (Bennett *et al.*, 1987). Owing to this transience, testing of multiple serum samples is often required to detect the galactomannan antigen (Andriole, 1993). Besides the presence of circulating galactomannan in the blood, other non-galactomannan antigens have been detected in sera and urine of animals or humans with invasive aspergillosis (Haynes *et al.*, 1990; Yu *et al.*, 1990; Philip and Radigan, 1989). This paper reports the use of a biotin-avidin amplified sandwich ELISA (BALISA) which detects protein antigens in circulation of experimental invasive aspergillosis of rabbits.

MATERIALS AND METHODS

Microorganism. *Aspergillus fumigatus* M175/85 used in this study was isolated from a tracheal aspirate of a diabetic patient with pulmonary abscesses.

Inoculum preparation. Conidia of *A. fumigatus* M175/85 were harvested by washing 6 day old Malt Agar cultures of *A. fumigatus* with 0.1% Tween 80 made up in 0.8% NaCl. The fungal suspension was filtered aseptically through 4 layers of sterile gauze and vortexed vigorously to obtain a suspension of singly dispersed conidia of *A. fumigatus*. The concentration of viable conidia was determined by culturing in duplicates, diluted conidial suspension onto Sabouraud Dextrose Agar (Difco) for 24-48h at 30°C. Once early growth as evidenced by pin-point filamentous colonies was seen, the colonies from two plates were counted, averaged and expressed as colony forming units per milliliter (cfu/ml) taking into consideration the dilution factor. The amount of viable conidia remained stable for at least a week at 4°C.

Rabbit model. Tricolor rabbits and imported New Zealand White (NZW) rabbits were used in this study. The Tricolor rabbits which were locally bred were obtained from the Animal House of the Institute for Medical Research, Kuala Lumpur. Sixteen Tricolor rabbits were divided into 3 groups: A1 (6 rabbits), A2 (5 rabbits) and A3 (5 rabbits). Rabbits in group A1 and A2 were injected intravenously via the marginal ear vein with 1×10^6 and 1×10^7 conidia respectively. Rabbits in group A3 were injected intravenously with the diluent (0.1% Tween 80 in 0.8% NaCl) used to make the conidial suspension. Nineteen NZW rabbits were divided into 4 groups: B1 (6 rabbits), B2 (6 rabbits), B3 (4 rabbits) and B4 (3 rabbits). Rabbits in groups B1, B2 and B3 were injected intravenously with 1×10^6 , 1×10^7 and 1×10^8 conidia respectively. Rabbits in group B4 served as the control and was injected as described for rabbits in group A3. Rabbit number 13 and 15 were eventually excluded from the study as they failed to develop invasive aspergillosis. Pre-infection serum was collected 1-2 days prior to infection with viable conidia. Following intravenous inoculation with viable

conidia, rabbits were bled daily until rabbits died. Blood collected were separated from the clot and used in the detection of *Aspergillus* antigens. At necropsy, 5 organs (liver, kidney, spleen, heart and lung) were cultured for *A. fumigatus*. Samples of tissues from each organ were macerated (Ultra Turax, Germany) using aseptic techniques and cultured in duplicates onto Sabouraud Dextrose Agar (Oxoid) at 37°C for 1 week.

The BALISA. The BALISA is a double antibody sandwich ELISA which is amplified with biotin-avidin (Abdul Samad *et al.*, 2001). The BALISA utilizes capture and detector polyclonal antibodies raised against water-soluble (WS) mycelial antigens of *A. fumigatus* in rabbits and guinea pigs respectively. Briefly, following an overnight coating with capture antibody (rabbit anti-WS antibodies) at 4°C, non-specific binding sites were blocked by incubating wells with 5% bovine serum albumin (BSA) for 15 min at 37°C. Wells were next incubated with control and test sera for 45min. This was followed by the incubation with the detector antibody (guinea-pig anti-WS antibodies) for 1h. Following this, 1h incubation period each were conducted in succession with a 1:40,000 dilution of anti-guinea pig antibody conjugated with biotin (Sigma) and a 1:200,000 dilution of avidin-peroxidase (Sigma). Next 0.01% orthophenylenediamine (OPD) dissolved in citrate-phosphate buffer containing 0.012% H₂O₂ were added and incubated for 30min. At the end of the 30min period, the reaction was stopped by the addition of 2N HCl and absorbance was read using an ELISA plate reader (Dynatech MR 5000, USA) set at a wavelength of 490nm. The capture and detector antibodies were used at concentrations of 1.38µg/ml and 1.11µg/ml respectively. All incubations were carried out at 37°C in a water-bath. The total duration of the BALISA following an over-night coating step took 4h and 30min. Every plate consisted of substrate, conjugate, positive and negative controls. Negative control consisted of pooled normal rabbit sera whilst positive control consisted of 0.13µg/ml of WS antigen diluted in pooled normal rabbit sera. Controls were incubated in quadruplicates whilst test sera were incubated in duplicates.

Standard Curve. Known concentrations of WS antigens were diluted 2 fold serially in pooled normal rabbit serum to obtain WS antigen concentrations ranging from 50,000ng/ml to 2ng/ml. These standards were included in every experiment.

Concanavalin A Affinity Chromatography. Concanavalin A chromatography was conducted in mini disposable columns (Spectrum, USA) according to the technique of Wilson and Hearn 1983. A column (12.3cm x 1.8cm) of Con A-Sepharose (Amersham, UK) was equilibrated with 1mM Tris-HCl buffer, pH 7.2 containing 1mM CaCl₂, 1mM MnCl₂ and 0.25M NaCl. The WS antigen was dialysed against

this buffer and any insoluble material removed by centrifugation at 100,000g. Two milliliters of the supernatant of 5.0mg/ml protein concentration was applied to the column and the protein-rich unbound fraction (UBF) was collected in 200µl aliquots in eight eppendorf tubes. Following this, 2ml of 0.2M alpha methylmannoside (Sigma) prepared in 1 mM Tris-HCl buffer resulted in elution of the carbohydrate-rich bound fraction (BF). Six fractions of BF of 200µl each were collected. The BF and UBF were tested with the BALISA and used in immunoblotting studies.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using a mini gel system (Mighty Small II SE 250, Hoefer Scientific Instruments). Electrophoresis was conducted using a discontinuous buffer system of Laemmli, 1970. Antigens were separated on 10% (T) polyacrylamide gels containing 0.375M Tris-HCl pH 8.8, 5% (v/v) glycerol, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.05% TEMED. Stacking gels used 4% (T) polyacrylamide which consisted of 0.125M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.08% (w/v) ammonium persulfate and 0.1% TEMED. Samples for analysis were treated in equal volumes with treatment buffer with final concentrations of 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.01% (w/v) of bromophenol blue made up in 0.125M Tris-HCl pH 6.8. Samples were boiled for 3min, cooled and 10µl samples were placed into wells. Tank buffer consisted of 0.025M Tris, 0.192M glycine and 0.1% SDS. Wide range molecular weight markers (20-205kD) (Sigma) of the same volume as the samples were aliquoted directly into wells without prior treatment. Electrophoresis was conducted at constant current of 45 mA with constant electrophoretic temperature at 15°C (Multitemp Thermostatic Cooler, Hoefer Scientific Instruments) for 45min.

Electroblot. Antigens separated by SDS-PAGE were transferred to nitrocellulose membranes (Micro Filtration Systems) using the mini transblot system (TE Transphor Electrophoresis Unit, Hoefer Scientific Instrument). Nitrocellulose membranes were placed on the anode side, whilst the gel was placed on the cathode side of the cassette. Transblot was conducted in transfer buffer (0.025M Tris, 0.192M glycine, 20% methanol, 0.1% SDS) at constant current of 140mA for 3h.

Immunoblot. Immunoblotting was conducted with a slight modification in technique as described previously (Abdul Samad *et al.*, 1993b). Following transblot, the nitrocellulose membrane was soaked in 0.01M phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 and 5% Skim Milk (Difco) (SMPBST) for 1h at 37°C and overnight at 4°C. After three 10min washes with 0.01MPBST, the nitrocellulose membrane was incubated with detector antibody at 37°C for 2h with agitation. The detector antibody which consisted

of anti-WS antibodies raised in rabbits was diluted 1: 200 with SMPBST containing 0.1% Skim Milk. The diluted blocking buffer was also used to make dilutions of other antibodies and reagents. Following washing, the blot was subsequently incubated with a 1:1000 dilution of anti-rabbit peroxidase conjugate (Sigma) at 37°C for 1h. The membrane was washed again and the antigen-antibody complexes were localized by staining for peroxidase activity with a commercial substrate containing a mixture of diaminobenzidine, 4-chloro-1-naphthol and hydrogen peroxide (Pierce Laboratories, USA). The reaction was stopped by washing the blots with distilled H₂O once the bands appeared clear. This usually took 15min.

RESULTS

Experimental Invasive Aspergillosis In Rabbits

i) Mortality rates of rabbits. Two species of rabbits i.e. Tricolor and New Zealand White were inoculated intravenously with various inoculum of viable *A. fumigatus* conidia in order to establish an invasive aspergillosis model. Tricolor rabbits inoculated with 1 x 10⁶ conidial demonstrated 16.7% mortality by day 2 post-infection (Figure 1). The mortality rates increased to 83% by day 5 post-infection and attained 100% mortality by day 6 post-infection. A ten fold increase in inoculum size in Tricolor rabbits resulted in more severe infection as seen by a 80% mortality at day 2 post-infection and 100% mortality by day 3 post-infection. In contrast to Tricolor rabbits which demonstrated mortality when inoculated with 1 x 10⁶ conidia, NZW rabbits inoculated with the same inoculum size remained healthy and survived the entire period of observation. Similarly, NZW rabbits inoculated with 1 x 10⁷ conidia demonstrated a slower progression of infection than Tricolor rabbits inoculated with the same amount of conidia. At day 2 post-infection, the mortality was 25% and 100% mortality was attained five days following infection. New Zealand Rabbits inoculated with 1 x 10⁸ conidia also demonstrated a slower progression of infection than Tricolor rabbits inoculated with a ten fold lower inoculum. Both groups of rabbits achieved 100% mortality by day 3 post-infection. However, at day 2 post-infection a 55% decrease in mortality was seen in the NZW rabbits as compared to Tricolor rabbits.

ii) Isolation of A. fumigatus from organs of infected rabbits. Tricolor rabbits infected with 1 x 10⁶ conidia/ml demonstrated *A. fumigatus* isolation rates of 83.3%, 100% and 83% from liver, kidney and spleen respectively. Lower isolation rates of 50% each was seen for both the heart and lung (Table 1). When a 10 fold higher inoculum was used to infect the rabbits, a marked increase of 50% in isolation rates was seen for the heart and lung. An overall higher isolation rate of *A. fumigatus* of 96% (24 of 25 organs cultured) was seen in rabbits

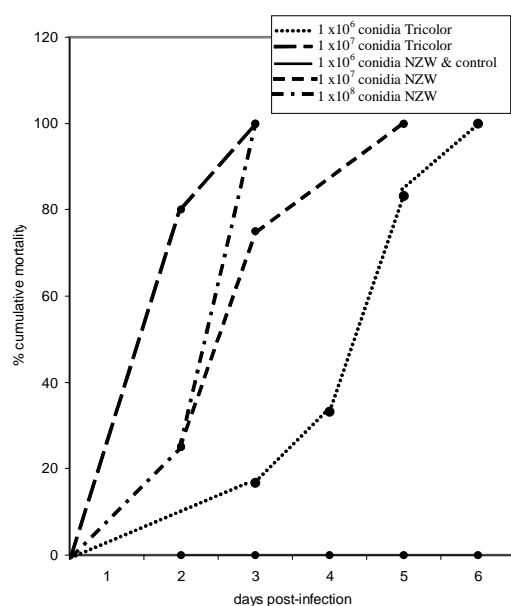


Figure 1. Mortality rates of Tricolor and New Zealand White rabbits inoculated intravenously with *Aspergillus fumigatus*.

inoculated with 1×10^7 conidia as opposed to 73% (22 of 30) seen in rabbits inoculated with 1×10^6 conidia. None of the 30 organs cultured for NZW rabbits inoculated with 1×10^6 conidia yielded *A. fumigatus* growth. Isolation rates in NZW rabbits inoculated with 1×10^7 and 1×10^8 conidia were lower by 46 and 56 % respectively as compared to Tricolor rabbits infected with 1×10^7 conidia.

BALISA

i) Template Absorbance Values. Table 2 and 3 demonstrates the template absorbance values and standard deviations for WS antigens and normal rabbit sera of Tricolor and NZW rabbits. The positive control consists of 0.13ug/ml of WS antigen reconstituted in normal rabbit serum. The negative control consisted of pooled normal rabbit sera. In three separate trials for Tricolor rabbits, the absorbance readings for the negative control was 0.402, 0.396 and 0.407 (Table 2). The mean absorbance of these was 0.402 with a mean standard deviation of 0.030. The mean absorbance value was comparable to the mean absorbance value of 16 individually tested normal rabbit serum of 0.388 whilst the value of the standard deviation was 0.060. The mean absorbance value of the positive control from three trials was 0.931 with a standard deviation of 0.027. All absorbance readings for NZW rabbits were much lower than the absorbance readings for Tricolor rabbits (Table 2). The mean absorbance value for pooled normal rabbit sera of 0.250 was comparable to the mean absorbance reading for the 17 individually tested rabbit sera which was 0.218. The standard deviation of the former and the latter were 0.005 and 0.060

respectively. In order that the absorbance values from different tests can be compared, all readings were adjusted to the template absorbance values obtained in Table 2 and 3. The adjustments were made by multiplying a ratio to all the absorbance values obtained in other tests. The ratio was calculated as follows:

$$\frac{\text{Absorbance of PCS in the template}}{\text{Absorbance of PCS in each assay}}$$

ii) Determination of the Threshold Absorbance Value and the Minimum Detectable Concentration (MDC). Since the absorbance values of the 16 and 17 individually tested normal rabbit sera from Tricolor and NZW rabbits respectively showed a normal distribution (data not shown), the threshold absorbance value was taken as the mean absorbance value of the negative control in each test + 2SD. The threshold absorbance value for 2 trials for Tricolor rabbits was 0.318 and 0.442 with a mean value of 0.370 (Table 4). The minimum detectable concentration (MDC) of WS antigens was determined as the lowest concentration of WS antigen that shows an absorbance value greater or equal to the threshold absorbance value determined in each assay. Based on this interpretation the mean MDC for Tricolor rabbits was 0.008 μ g/ml. The mean threshold absorbance value from 3 trials for NZW was 0.182 and the mean MDC was 0.013 μ g/ml (Table 4).

iii) Detection of Aspergillus antigens infected rabbit. The results of antigen detection in Tricolor rabbits (groups A1-A3) and NZW rabbits (B1-B4) are shown in Table 5 and 6. All 11 Tricolor rabbits inoculated with 1×10^6 and 1×10^7 conidia/ml demonstrated antigenemia. Seventy-five percent of the 24 sera tested from these two groups of rabbits were antigen positive. Antigen detection rates were lower in NZW rabbits. None of the 60 serum samples obtained from NZW rabbits inoculated with 1×10^6 conidia demonstrated antigenemia (Table 6). New Zealand White rabbits inoculated with 1×10^7 and 1×10^8 conidia demonstrated antigenemia in 50% (2 of 4 rabbits) and 75% (3 of 4) of rabbits respectively. Additionally, *Aspergillus* antigens were present in only 36% of the 14 serum samples tested from these rabbits. Initial antigenemia was seen at day one post-infection in 33.33% of Tricolor rabbits inoculated with 1×10^6 conidia. On the other hand, initial antigenemia was seen in 80% of Tricolor rabbits inoculated with 1×10^7 conidia. The duration of antigenemia was seen for 1 to 5 days and 1 to 3 days post-infection in Tricolors inoculated with 1×10^6 and 1×10^7 conidia respectively. In contrast, in NZW rabbits, initial antigenemia at day 1 post-infection was only seen in 1 of the 5 rabbits which were antigen positive. The concentration of antigens detected in Tricolor rabbits ranged from 374 to 1172ng/ml whilst in NZW rabbits lower antigen concentrations of 204 to 472ng/ml were obtained. A rabbit

Table 1. Isolation of *Aspergillus fumigatus* obtained from Tricolor and New Zealand White rabbits inoculated intravenously with *A. fumigatus* conidia

Rabbits	Inoculum (conidia)	Organ cultured positive					Total positive organ Total organ positive(%)
		Organs cultured (%)					
		Liver	Renal	Spleen	Heart	Lung	
Tricolor	1x 10 ⁶	5/6 (83.3%)	6/6 (100%)	5/6 (83 %)	3/6 (50 %)	3/6 (50 %)	22/30 (73%)
	1x10 ⁷	5/5 (100%)	5/5 (100%)	4/5 (80%)	5/5 (100%)	5/5 (100%)	24/25 (96%)
NZW	1x 10 ⁶	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/30 (0%)
	1x10 ⁷	3/4 (75%)	2/4 (50%)	2/4 (50%)	2/4 (50%)	1/4 (25%)	10/20 (50%)
	1x10 ⁸	2/4 (50%)	0/4 (0%)	4/4 (100%)	2/4 (50%)	0/4 (0%)	8/20 (40%)

Table 2. Mean absorbance value and standard deviation of water soluble antigens and 16 normal Tricolor rabbit sera tested with BALISA

Trial	PCS ^a	SD	NCS ^b	SD	NRS ^c	SD ^d
1	0.927	0.029	0.402	0.035	0.391	0.06
2	0.935	0.026	0.396	0.030	0.387	0.06
3	0.931	0.026	0.407	0.026	0.386	0.06
Mean	0.931	0.027	0.402	0.030	0.388	0.06

^a PCS, Positive control sera of 0.13 µg/ml WS antigen prepared in pooled normal rabbit sera of Tricolor rabbits.

^b NCS, Negative control sera of pooled normal rabbit sera of 16 Tricolor rabbits.

^c NRS, Normal rabbits sera of 16 Tricolor rabbit sera which tested individually.

^d Standard deviation

Table 3. Mean absorbance value and standard deviation of water soluble antigens and normal sera of 17 New Zealand White rabbit sera tested with BALISA

Trial	PCS ^a	SD	NCS ^b	SD	NRS ^c	SD ^d
1	0.442	0.026	0.251	0.005	0.217	0.05
2	0.424	0.030	0.250	0.006	0.221	0.05
3	0.420	0.025	0.248	0.005	0.217	0.05
Mean	0.422	0.027	0.250	0.005	0.218	0.05

^a PCS, Positive control sera of 0.13 µg/ml WS antigen prepared in pooled normal rabbit sera of New Zealand White rabbits.

^b NCS, Negative control sera of pooled normal rabbit sera of 17 New Zealand White rabbits.

^c NRS, Normal rabbits sera of 17 New Zealand White rabbit sera which were tested individually.

^d Standard deviation

from the control un-inoculated Tricolor group was positive for 2 consecutive serum samples tested, whilst a single serum from a NZW rabbit from the control group was also positive. The overall sensitivity of antigen detection in Tricolor and NZW was 84.2% (16 of 19 rabbits), whilst the specificity was 85.7% (12 of 14 rabbits) (Table 7).

iv) Characterization of water-soluble antigens. Fractions which demonstrated strong immunoreactivity when tested with the in-house BALISA were only fractions 6 to 8 of the UBF demonstrating absorbance ratio values of 3.0 to 4.0. However, all BF fractions failed to show positive results for the BALISA (absorbance ratio values of less than 1.5) (Figure 2). Fractions 6 to 8 of the UBF which showed strong immunoreactivity also demonstrated higher protein concentrations of 500 - 1900µg/ml when compared to other unbound fractions. Electrophoresis of WS antigens revealed at least 10 to 13 distinctive antigen bands which ranged from less than < 20 - 116kD (data not shown). Electrophoresis

of BF and UBF resulted in far more protein bands for the UBF (data not shown).

v) Immunoblot of WS antigens and WS antigens fractionated with Concanavalin A. Immunoblot studies revealed at least 13 immunoreactive bands for WS antigens ranging from 22-138kD (lanes 8 and 9, Figure 3). Strong immunoreactive bands were seen in regions of 73-85kD, 66-67kD and 38-46kD for WS antigens. Weak immunoreactive bands ranging from 22 to 105kD were detected for only fraction 6 (lane 7) of the BF. Fractions 6 to 8 (lane 2 to 9) of UBF revealed clear immunoreactive regions of 17-22kD and 52-63kD (Figure 4). Less clear immunoreactive regions of 38-46kD was also seen. Common immunoreactivity in the regions of 38 - 46 kD of descending clarity was seen for WS, UBF, BF.

DISCUSSION

Table 4. Minimum detectable concentration of water soluble antigens in sera of Tricolor and New Zealand White rabbits tested with the BALISA.

Rabbits	Trial	Mean threshold absorbance value ^a	MDC ^b (µg/ml)
Tricolor	1	0.318	0.008
	2	0.442	0.008
	Mean	0.370	0.008
New Zealand Whites	1	0.151	0.016
	2	0.210	0.016
	3	0.221	0.008
Mean		0.182	0.013

^a Mean threshold absorbance value was taken as the mean absorbance value of the negative control in each test plus 2SD

^b MDC, Minimum detectable concentration was the lowest concentration of water soluble antigen that showed an absorbance value greater or equal to the threshold value.

Experimental invasive aspergillosis was established in rabbits in order to test the utility of the in-house antibody sandwich ELISA amplified by biotin-avidin system. Rabbits models of invasive aspergillosis was established by inoculating non-immunosuppressed Tricolor and NZW rabbits intravenously with various inoculum of viable *A. fumigatus* conidia.

The study demonstrated that the Tricolor rabbits were more susceptible to *Aspergillus* infection than the New Zealand Whites. Inoculum doses of 1×10^6 conidia achieved 100% mortality within 6 days in Tricolor rabbits and 73% of the organs cultured from these rabbits at necropsy were positive for *A. fumigatus*. In contrast, the same inoculum of viable conidia failed to establish invasive aspergillosis in NZW rabbits as rabbits remained healthy throughout the experimental period and all organs cultured were negative for *A. fumigatus*. The failure of NZW rabbits inoculated with 1×10^6 conidia to succumb to infection has also been shown by Dupont *et al.* (1987). In their study, it was shown that an inoculum size of less than 3×10^6 results in sublethal infections and that a 1×10^6 conidial inoculum can be used to immunize rabbits.

In a previous study by Abdul Samad *et al.* (1993a) using Tricolor rabbits to establish invasive aspergillosis demonstrated that they were far less susceptible to infection than the ones used in this study. In the previous study, the 12 Tricolor rabbits infected intravenously with 1×10^6 conidia of the same strain of *A. fumigatus* remained healthy and did not succumb to infection. However, liver, kidney, spleen, heart and lung of some of the rabbits were culture positive for *A. fumigatus*. The differences in susceptibility of Tricolor rabbits tested at different times may reflect the inherent genetic variations in rabbits within the same species. Possible reduction in virulence of *A. fumigatus* can be ruled out as conidia was made up fresh from lyophilized stock.

The severity of infection in rabbits was dependent on the amount of conidial inoculum administered to the rabbits.

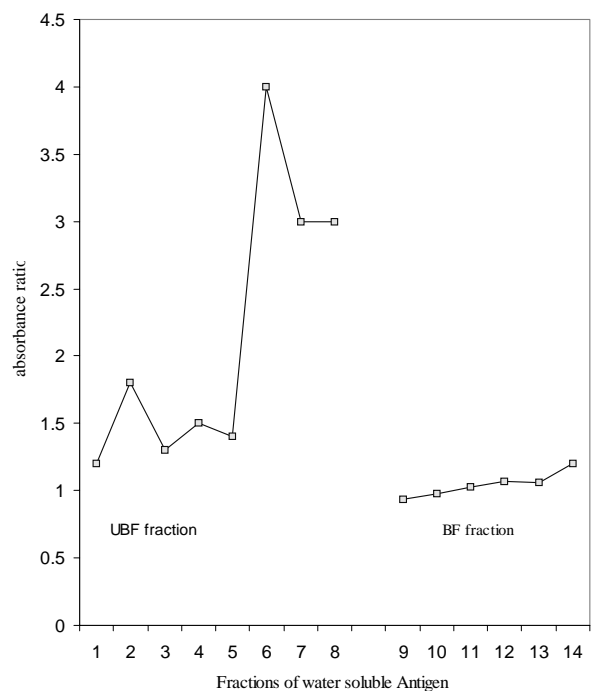


Figure 2. Reactivity of unbound (UBF) and bound (BF) fractions of water soluble antigen of *Aspergillus fumigatus* with BALISA

Tricolor rabbits inoculated with 1×10^7 conidia demonstrated 100% mortality at day 3 post-infection and a 96% isolation rate of *A. fumigatus* from organs. In contrast, Tricolor rabbits inoculated with a ten fold lower inoculum demonstrated 100% mortality 3 days later and a decrease in isolation rates by 23%. Similarly, NZW rabbits inoculated with 1×10^8 conidia achieved 100% mortality 2 days earlier than NZW rabbits inoculated with 1×10^7 conidia. The relation of increased severity of infection with increases in inoculum sizes has also been shown by other investigators (de Repentigny *et al.*, 1991; Yu *et al.*, 1990; Abdul Samad *et al.*, 1993a)

Antigen in sera was detected in all 11 Tricolor rabbits inoculated with 1×10^6 and 1×10^7 conidia. However, lower antigen detection rates of 50% (2 of 4 rabbits) and 75% (3 of 4 rabbits) were obtained for NZW inoculated with 1×10^7 and 1×10^8 conidia respectively. The lower rates of antigenemia in NZW rabbits can be attributed presumably to the lower fungal burden in organs in these rabbits as evidenced by the isolation of *A. fumigatus* in only 40-50% of organs cultured. White *et al.*, (1977) demonstrated a trend for detecting antigens in sera of mice with higher fungal load in tissue than in mice with lower fungal load.

In our rabbit model of invasive aspergillosis, circulating aspergillus antigens first appeared at day 1 post-infection in about a third and 80% of Tricolor rabbits inoculated with 1×10^6 and 1×10^7 conidia/ml respectively. In contrast, initial antigenemia a day following infection was seen only in a single NZW rabbit which was inoculated with 1×10^8 conidia.

Table 5. Detection of *Aspergillus* antigens in Tricolor rabbits inoculated with *Aspergillus fumigatus* conidia using the BALISA.

Group	Rabbit No.	Inoculum ^a (conidia)	Concentration (ng/ml) of <i>Aspergillus</i> antigens on day post-infection:						
			1	2	3	4	5	6	
A1	6	1x 10 ⁶	0	NT ^b	522	NT(D) ^c			
	7	"	374	NT	574	1172	453(D)		
	8	"	384	NT	516	446	537(D)		
	9	"	0	NT	NT	487	TL(D)		
	10	"	0	NT	774(D)				
	11	"	0	NT	536	473	0	NT(D)	
A2	12	1x 10 ⁷	397	NT(D)					
	13	"	0	NT	1048(D)				
	14	"	431	NT	D				
	15	"	488	NT	D				
	16	"	337	NT	D				
A3	1	Control	0	0	0	NT	0	NT	
	2	"	NT	318	343	0	0	0	
	3	"	NT	0	0	0	0	0	
	4	"	0	NT	0	NT	0	0	
	5	"	0	NT	0	0	0	0	

^a Conidia given intravenously; ^b NT, Not tested; ^c D, Rabbit died

Table 6. Detection of *Aspergillus* antigens in New Zealand White rabbits inoculated with *Aspergillus fumigatus* conidia using the BALISA

Group	Rabbit No.	Inoculum ^a (conidia)	Concentration (ng/ml) of <i>Aspergillus</i> antigens on day post-infection:														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	
B1	4	1x10 ⁶	0	NT ^b	0	0	0	0	0	0	0	NT	0	0	0	0	0
	5	"	0	NT	0	0	0	0	0	0	0	NT	0	0	0	0	0
	6	"	0	NT	0	0	0	0	0	0	0	NT	0	0	0	0	0
	7	"	0	NT	0	0	0	0	0	0	0	NT	0	0	0	0	0
	8	"	0	NT	0	0	0	0	0	0	0	NT	0	0	0	0	0
	9	"	0	NT	0	0	0	0	0	0	0	NT	0	0	0	0	0
B2	10	1x 10 ⁷	0	NT(D) ^c													
	11	"	0	NT	316(D)												
	12	"	0	NT	0(D)												
	14	"	0	NT	472(D)												
B3	16	1x 10 ⁸	334	NT	NT(D)												
	17	"	0	NT	209(D)												
	18	"	0	NT(D)													
	19	"	0	204(D)													
B4	1	Control	0	TL	0	0	0	0	0	0	0	NT	0	0	0	0	0
	2	"	0	TL	0	0	0	0	0	0	411	NT	0	0	0	0	0
	3	"	0	TL	0	0	0	0	0	0	0	NT	0	0	0	0	0

Table 7. The sensitivity and specificity of the BALISA in detecting *Aspergillus* antigenemia in rabbits with invasive aspergillosis

Rabbits	Sensitivity ^a	Specificity ^b
Tricolor	11/11 (100%)	4/5 (80%)
New Zealand White	5/8 (62.5%)	8/9 (88.8%)
Overall	16/19 (84.2%)	12/14 (85.7%)

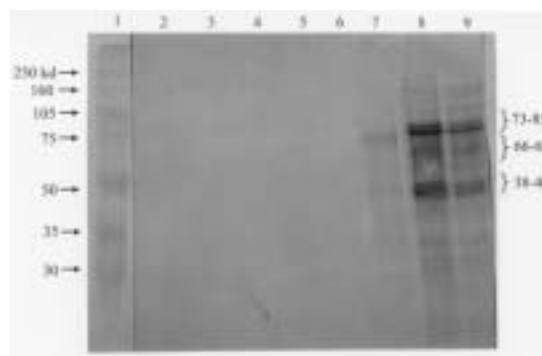
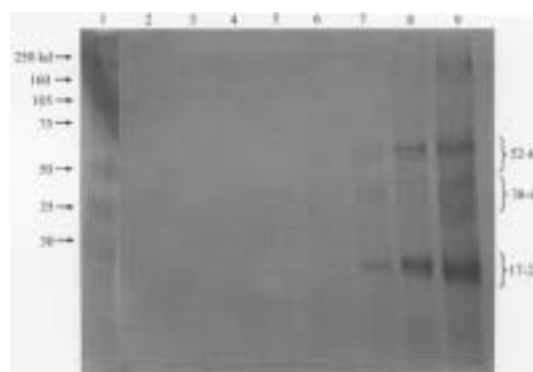
^a Percentage of rabbits with invasive aspergillosis which demonstrated antigenemia

^b Percentage of rabbits without invasive aspergillosis which did not demonstrate antigenemia

Most reports using rabbit models of IA have reported earliest antigen detection at 48h following infection (Lehmann and Reiss, 1978; Sabetta *et al.*, 1985; de Repentigny *et al.*, 1987). A previous study conducted in our laboratory which used the same capture and detector antibody for a double antibody sandwich ELISA without biotin-avidin amplification demonstrated initial antigen detection at day 2 post-infection in immunosuppressed rabbits infected with *A. fumigatus* (unpublished data). The reason for earlier initial detection of antigens in the present study could be attributed to the particularly increased susceptibility of this group of Tricolor rabbits to *Aspergillus* infection and the fact that a more sensitive ELISA was employed in the present study to detect *Aspergillus* antigens.

Tricolor rabbits inoculated with 1×10^6 conidia demonstrated antigenemia 1-5 days following infections whilst antigenemia for Tricolor rabbits inoculated with 1×10^7 conidia was seen 1-3 days post-infection. The short durations of antigenemia in experimental models in rabbits of 2-3 days Patterson *et al.* (1988), 2-4 days (Sabetta *et al.*, 1985; de Repentigny *et al.*, 1987) and 2-6 Dupont *et al.* (1987) days post-infection have been reported. In our study, antigenemia was normally seen in rabbits with lethal infection. In about 81% of 16 rabbits positive for antigens, antigens were detected 2 or less days prior to death. This finding is in agreement with other investigators who have reported antigenemia in lethally infected rabbits shortly before death (Sabetta *et al.*, 1985; Dupont *et al.*, 1987; Repentigny *et al.*, 1987).

The overall sensitivity and specificity of the BALISA in detecting antigens for both Tricolor and NZW rabbits were 84.2% and 85.7% respectively. Even though the limit for antigen detection for BALISA ranged from 8 to 13ng/ml, all antigens levels detected were high ranging from 204 to 1172ng/ml in all rabbits. It can be presumed then that all negative sera had antigen concentrations lower than the threshold antigen detection level. A biotin-streptavidin double antibody sandwich ELISA Fujita *et al.* (1988) demonstrated a sensitivity of 89% (16 of 18) and a specificity of 99% in detecting carbohydrate antigens in circulation of proven cases of invasive aspergillosis. They reported a

**Figure 3.** Immunoblot of Concanavalin A bound fractions (BF) and water soluble (WS) antigens of *Aspergillus fumigatus*. Lanes 2 to 7: fractions 1 to 6 of BF, lanes 8 to 9: WS antigens of 1:8 and 1:32 dilution respectively, lane 1: high molecular weight rainbow marker.**Figure 4.** Immunoblot of Concanavalin A unbound fractions (UBF) of *Aspergillus fumigatus*. Lanes 2 to 9: fractions 1 to 8 of UBF, lane 1: high molecular weight rainbow marker.

minimum antigen detection limit of 1.6ng/ml and patients demonstrated antigen levels ranging from 2 to 17ng/ml. The differences in antigen detection limits, ranges in antigen concentration detected and ultimately the sensitivity and specificity of our sandwich ELISA to that of Fujita *et al.* (1988) may be attributed to the different type of capture and detector antibodies used (polyclonal anti-WS mycelial antigens); the different types of antigens detected (protein) and the different species (rabbits) used for antigens detection used in our study. The ELISA with the lowest antigen detection limit (0.5 to 1ng/ml) to date is the Platelia *Aspergillus* which is a direct double antibody sandwich ELISA utilizing monoclonal antibodies to the galactomannan antigen in sera. The sensitivity and specificity of this ELISA is reported to be 90%-93% and 84%-95% respectively in patients with invasive aspergillosis (Verweij *et al.*, 1995; Maertens *et al.*, 1999).

Antigenemia in rabbits with experimental IA have been detected using inhibition ELISAs (Sabetta *et al.*, 1985; de Repentigny *et al.*, 1987; Dupont *et al.*, 1987). Sabetta *et al.*

(1985) detected antigens in 83.3% of rabbits but not in the control uninfected group. The antigen detection limit of their inhibition ELISA was 5 to 10ng/ml and the antigen concentrations ranged from 5 to 80ng/ml. Repentigny *et al.* (1987) detected antigens in serum of all 4 rabbits with invasive aspergillosis and reported a threshold antigen detection level of 10ng/ml. In contrast, Dupont *et al.* (1987) detected antigenemia in only 33.3% of 12 infected rabbits with concentrations ranging from 108 to 356ng/ml.

The majority of immunoassays used to detect antigenemia in invasive aspergillosis have detected a heat stable carbohydrate antigens (Sabetta *et al.*, 1985; de Repentigny *et al.*, 1987; Dupont *et al.*, 1987) or galactomannan (Rogers *et al.*, 1990; Verweij *et al.*, 1995). Since these carbohydrate or galactomannan antigens are usually in immune-complexes, sera are usually treated using weak acids and or heat prior to antigen detection as this increases the sensitivity of antigen detection. In our study heating of normal sera spiked with WS antigens resulted in reduction of absorbance values of up to 47%. This suggests that the antigen detected by the BALISA is probably a heat labile proteinaceous antigen. This suggestion was strengthened by the finding that only the protein rich (UBF) component of the Concanavalin A fractionated WS antigen reacted strongly with the BALISA. All carbohydrate fractions (BF) of the WS antigen failed to react. In addition, in immunoblot studies, clear immunoreactive regions were seen for the UBF and not for the BF. Concanavalin A is a lectin which has affinity for carbohydrates with glucopyranosyl and mannopyranosyl residues (Wilson and Hearn, 1983). The fraction of the WS antigen which binds and does not bind concanavalin-A are the carbohydrate bound fraction and the protein rich unbound fraction respectively.

In immunoblotting studies the antisera used to probe the electrophoresed antigens consisted primarily of the capture antibody (rabbit anti-WS antibodies) used in the BALISA. The WS antigens showed three areas of prominent immunoreactivity of 38-46kD, 66-68kD and 73-85kD regions. The UBF was more immunoreactive than the BF and showed strong immunoreactivity at regions of 17-22 and 52-60kD. A common immunoreactive region of 38-46kD which was most distinct for the WS antigens were also seen but less distinctly for the UBF and BF. Hearn *et al.*, (1990) demonstrated 3 prominent immunoreactive regions of 19kD, 38-51kD and 78-100kD when WS antigens from *A. fumigatus* were reacted with antisera raised against WS antigens. Like our study, similar common immunoreactive regions of 38-51kD was seen for the WS, UBF and BF tested. However, in their study, the BF was found to be far more immunoreactive than the UBF. In a previous study of immunoblotting WS antigens of *A. fumigatus* with polyclonal *Aspergillus* antisera, major immunoreactive bands were detected at 36kD and 69-69.5kD with a broad diffuse area of immunoreactivity at 50-60kD (Abdul Samad *et al.*, 1993b).

Mathew *et al.*, (1985) who analysed the serologic response of patients with invasive aspergillosis showed that 81% of these patients reacted to a 40kD component. Another study (de Repentigny *et al.*, 1991), of experimental invasive aspergillosis showed seroconversion against UBF antigens of 41, 54 and 71kD. Amongst these three antigens, seroconversion occurred most frequently to the 41kD antigen. Whether or not the immunoreactive antigens detected in our study are the same as those reported by other investigators is not known at this point in time.

In this study, invasive aspergillosis models were established in two types of rabbits using various concentrations of fungal conidia. This was conducted in order to procure a rabbit model which would be suitable for evaluating the BALISA. A suitable model would be one which has a high enough degree of infection which allows for release of antigens into the circulation for a sustained period of time. This infection must also not be so overwhelming so as to result in early death of rabbits. Tricolor rabbits inoculated with 1×10^6 conidia demonstrated this criterion as sustained antigenemia and later death of rabbits was seen in only this group of rabbits.

This study reports the use of an indirect antibody sandwich ELISA amplified by a biotin-avidin and utilizing polyclonal antibodies raised towards WS antigens of *A. fumigatus*. The BALISA was found to be useful in detecting antigens in experimental invasive aspergillosis in rabbits. The antigens detected are thought to be protein in nature.

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