

Short communication

Highly sensitive detection of fungal antigens by ultrasound-enhanced latex agglutination

M. A. GRUNDY,* R. A. BARNES† & W. T. COAKLEY*

*School of Pure and Applied Biology, University of Wales College of Cardiff; and †Department of Medical Microbiology, University Hospital of Wales, Cardiff, UK

Treatment with ultrasound has been employed to greatly enhance the sensitivity of commercially available latex agglutination tests for fungal antigens. This 5 min procedure detects 40 pg ml^{-1} of *Candida albicans* mannan and 70 pg ml^{-1} of *Aspergillus fumigatus* galactomannan, a 250 and 500-fold improvement respectively over conventional agglutination test sensitivities. The ultrasound-enhanced test offers the possibility of improved diagnosis and management of patients with systemic candidosis or invasive aspergillosis.

Introduction

Autopsy proven evidence of fungal disease is found in up to 25% of organ transplant patients and patients with acute leukaemia. Systemic candidosis is the commonest mycosis seen, followed by invasive aspergillosis [1]. Invasive disease is mainly confined to severely immunocompromised individuals and neutropenic patients. Patients with a period of granulocytopenia exceeding three weeks have a 50% chance of developing invasive disease [2]. HIV patients are also susceptible. Mortality from invasive disease is very high and early diagnosis is of paramount importance in determining outcome. Establishing the diagnosis is difficult as the ante-mortem isolation of fungus from clinical specimens is rarely possible. Detection of antibodies is unreliable in immunocompromised patients, and seroconversion, if detected, often occurs late in the course of disease and is of prognostic significance with a very limited role in diagnosis.

It is recognized that a wide array of antigens circulate in systemic candidosis and invasive aspergillosis and reports of successful detection of antigenaemia appear regularly [3,4]. Many assay systems are based on the detection of mannan or galactomannan, a major cell wall component of *Candida* or *Aspergillus* spp. respectively. The characterization of immunodominant epitopes has led to the development of monoclonal antibodies that have been

incorporated into commercially available latex agglutination tests (LATs). Early studies with these LATs were encouraging [5,6], but evaluations with neutropenic patients showed that, although the positive predictive value of the tests was 53% in patients where serial samples were assayed, less than 12% of positive cases were detected from single serum samples [7]. This poor level of antigen detection is thought to be due to fluctuating levels of antigenaemia and the rapid clearance of mannan and galactomannan from the circulation. Strategies to improve the sensitivity of assays for the detection of fungal antigens are required to enable earlier diagnosis and better prognosis of patients.

Particles suspended in a non-cavitating ultrasonic standing wave field can become concentrated within seconds at positions separated by distances equal to half the acoustic wavelength [8,9]. This ultrasonic concentration phenomenon has previously been employed to significantly enhance both the rate [10–12] and, using diluted latex, the sensitivity [13; M. P. F. Gualano, unpublished results] of diagnostic agglutination tests. Through the use of ultrasound, the sensitivities of LATs for soluble C-reactive protein and particulate *Escherichia coli* antigens were improved $\times 256$ [13] and $\times 10\,240$ [M. P. F. Gualano, unpublished results] respectively as compared with standard test procedures. The objective of this study was to use ultrasound to increase the sensitivity of LATs for *Candida* and *Aspergillus* antigens and so offer the basis of a test for improved diagnosis of systemic candidosis and invasive aspergillosis.

Correspondence: Dr W. T. Coakley, School of Pure and Applied Biology, University of Wales College of Cardiff, PO Box 915, Museum Avenue, Cardiff, CF1 3TL, UK.

Table 1 *Candida albicans* mannan detection when dilutions of a positive control were mixed with different dilutions of antibody-coated latex and treated with ultrasound for 5 min

Dilution of mannan	Dilution of latex					
	1:1	1:2	1:4	1:8	1:16	1:32
1:1024	-	-	-	-	-	-
1:512	-	-	+*	-	-	-
1:256	-	-	+	+*	-	-
1:128	-	+*	+	+	+	-
1:64	-	+	+	+	+	+*
1:32	-	+	+	+	+	+
1:16	-	+	+	+	+	+
1:8	+*	+	+	+	+	+
1:4	+	+	+	+	+	+
1:2	+	+	+	+	+	+
1:1	+	+	+	+	+	+
Control	-	-	-	-	-	-

(+) Positive latex agglutination reaction, (-) negative reaction.
*Video-microscopy required for observation of agglutinates—all other positive (+) agglutination reactions visible to the naked eye.

Methods

Diagnostic kits

Pastorex *Candida* and Pastorex *Aspergillus* latex agglutination kits for the detection of soluble antigens of *Candida* and *Aspergillus* were purchased from Sanofi Diagnostics Pasteur S.A., France. Each kit included a suspension of antibody-coated latex particles, a positive antigen control (20 ng ml⁻¹ *Candida albicans* mannan or 75 ng ml⁻¹ *Aspergillus fumigatus* galactomannan), and dilution buffer, together with agglutination test cards (each imprinted with four circular test areas) and mixing sticks. These 'Pastorex' latex agglutination tests were the only kits commercially available in the UK which were directed against defined fungal antigens.

Conventional latex agglutination procedure

A series of doubling dilutions of the positive control from each of the above test kits was prepared in dilution buffer. Forty µl of each dilution of positive control was applied to one of a series of circles on the test cards. Ten µl of appropriate test latex was added to each circle and thoroughly mixed with the droplet of diluted positive control using a stick. The test card was then rocked at 160 rev. min⁻¹ on an agitator for 5 min at room temperature. Following agitation the reaction droplets were examined by eye for the greatest dilution of positive control which successfully agglutinated the latex. The dilution buffer was used as a negative test control.

Table 2 *Aspergillus fumigatus* galactomannan detection when dilutions of a positive control were mixed with different dilutions of antibody-coated latex and treated with ultrasound for 5 min

Dilution of galactomannan	Dilution of latex						
	1:1	1:2	1:4	1:8	1:16	1:32	1:64
1:2048	-	-	-	-	-	-	-
1:1024	-	-	-	+	-	-	-
1:512	-	-	+*	+	+*	-	-
1:256	-	-	+	+	+	+*	-
1:128	-	-	+	+	+	+*	-
1:64	-	+	+	+	+	+	-
1:32	-	+	+	+	+	+	+*
1:16	+	+	+	+	+	+	+
1:8	+	+	+	+	+	+	+
1:4	+	+	+	+	+	+	+
1:2	+	+	+	+	+	+	+
1:1	+	+	+	+	+	+	+
Control	-	-	-	-	-	-	-

(+) Positive latex agglutination reaction, (-) negative reaction.
*Video-microscopy required for observation of agglutinates—all other positive (+) agglutination reactions visible to the naked eye.

Ultrasonic test procedure

The ultrasonic apparatus employed has been previously described by Grundy *et al.* [13]. Reaction droplets comprising 10 µl of latex suspension and 40 µl of sample (diluted positive control or negative control) were held in a 2 mm i.d. glass capillary tube positioned so that the droplet was in the high acoustic pressure (axial) region of the standing wave field of a tubular-shaped ultrasonic transducer driven at 4.59 MHz. Each reaction droplet was exposed to ultrasound for 5 min with an applied transducer voltage of 50 V peak-to-peak. Following sonication, reaction droplets were expelled from the capillary, vigorously stirred with a mixing stick (to dissociate any particles which had been aggregated, but not actually agglutinated, in the ultrasonic standing wave field) and examined by eye or video-microscopy (see below) for latex agglutination.

The video-microscopy system employed [10] consisted of a light microscope with a video camera fitted to its monocular viewing head and a video monitor. For analysis (post-sonication and expulsion from the 2 mm i.d. capillary), a sample of each reaction droplet was drawn by capillarity into a 200 µm path-length, rectangular cross-section, glass microslide. Using the × 40 microscope objective the contents of each microslide were viewed on the video monitor. Each test with a particular dilution of test latex was compared with a negative control made up with that dilution of latex. Reactions were deemed to be positive for agglutination if particle clumps significantly larger than any seen in the negative controls were observed.

Table 3 Detection limits for *Candida albicans* mannan and *Aspergillus fumigatus* galactomannan

Fungal antigen	Conventional agglutination procedure	Ultrasound + latex dilution + video-microscopy	Increased sensitivity
Mannan	10 ng ml ⁻¹	40 pg ml ⁻¹	× 250
Galactomannan	37 ng ml ⁻¹	74 pg ml ⁻¹	× 500

Results and discussion

With the conventional test procedure, the greatest dilution of the stock *Candida* mannan or *Aspergillus* galactomannan giving agglutination visible to the naked eye was 1:2.

Through dilution of the test latex and/or treatment with ultrasound it was possible to detect agglutination with more dilute *Candida* mannan (Table 1). The most sensitive antigen detection was obtained with a 1:4 dilution of latex, where agglutination was visible to the naked eye or by video-microscopy at dilutions up to 1:256 and 1:512 respectively. The limiting mannan dilution for video-microscopy observation of agglutination contained clumps which were at least twice as wide as the occasional small (< 10 particles) clumps, seen at all latex dilutions, in sonicated negative controls.

The pattern of results for *Aspergillus* galactomannan detection through the use of diluted latex and/or treatment with ultrasound (Table 2) was similar to the results for *Candida* mannan detection. Here the most sensitive level of antigen detection (1:1024 dilution of galactomannan), seen by video-microscopy or the naked eye, was obtained using a 1:8 dilution of latex.

The positive detection limits for both the *Candida* mannan and *Aspergillus* galactomannan following sonication with diluted (1:4 and 1:8 for *Candida* and *Aspergillus* respectively) latex were confirmed by at least six replicate tests. Reported detection limits at other latex dilutions resulted from at least two tests.

As a specificity check, undiluted positive control from each kit was tested against undiluted latex from the other. Following sonication these reaction mixtures resembled negative test controls when viewed by eye or video-microscopy, i.e. no agglutination had occurred.

The small extent of video-microscopy enhancement of agglutination detection for the above polymeric fungal antigens contrasts with the great improvements observed with protein soluble-antigen [13] or with particulate antigens [M. P. F. Gualano, unpublished results].

The summary in Table 3 emphasizes the marked increase in the sensitivity of fungal antigen detection by the ultrasonic technique over the conventional LAT procedure. The 74 pg ml⁻¹ lower limit for galactomannan detection is also

a very significant improvement on the 7 ng ml⁻¹ limiting concentration previously reported for a galactomannan enzyme immunoassay [4,14]. In an evaluation of the *Aspergillus* galactomannan LAT employed here, the average number of serial samples required to obtain at least one positive test per patient (18/19 proven cases of aspergillosis) was 13.7 [14]. The greater sensitivity of the ultrasonic technique offers the possibility of fungal antigen detection from a reduced number of test samples and therefore earlier diagnosis and better prognosis of patients.

A programme is underway to reduce the technique to routine practice. It is unlikely that this development will result in a prohibitively expensive instrument.

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