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Anti-Cryptococcal-Globulin-Latex Production for Rapid Detection of *Cryptococcus neoformans* Polysaccharide Antigen in Cryptococcosis

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Abstract

Cryptococcosis has become the fourth leading life-threatening opportunistic infection in patients with AIDS, but also occurs in non-AIDS patients. In view of the increasing numbers of infection during last decade in Iran, use of rapid, sensitive and specific test for diagnosis of cryptococcal disease has become important than ever. We aimed to produce the reagents for latex cryptococcal antigen test. The antigen was prepared from NCPF 3168 strain of *Cryptococcus neoformans*. Anticapsular antiserum of *C. neoformans* raised in rabbits and latex carboxylate- modified beads were coated with antiserum. The maximally- reactive globulin dilution was obtained at dilution of 1:400. For evaluation of efficacy of reagents, challenged 38 BALB/C mice and other 38 mice were used as controls. The mice were bled and autopsied. Brain, heart and lung were checked by direct, histopathological and cultural examination for cryptococcosis. The sera from case and control mice were tested with Immunomycologic (Immy) kit and also our produced reagents (OPR) for detection of cryptococcal antigen. Moreover, 15 cerebrospinal fluid and 15 serum samples from patients with cryptococcal meningitis, 30 with aspergillosis, 30 with suspected other fungal infections, and 30 from healthy individuals were tested as well. The results showed that the sensitivity (97.3%) and specificity (100%) of OPR was quite comparable with those of Immy kit . Therefore, it could be regarded as a substitute for commercial kits.

Keywords: *Antigen detection, Anticryptococcal globulin, Latex agglutination, Cryptococcosis.*

Introduction

Cryptococcosis is a systemic fungal infection with a worldwide distribution and caused by the *C.neoformans*. Most common diagnosed clinical feature of disease is meningitis and meningoencephalitis. Recently the numbers of cases of cryptococcosis have been increased in Iran (1, 2 and our laboratory data). As the clinical symptoms are non-specific the paraclinical tests are needed for diagnosis.

India ink smears can not be used to definitely - the presence of *C.neoformans* but they can provide valuable support for presumptive diagnosis that guides the direction of further diagnostic efforts. When the test is performed in this manner, *Cryptococci* are seen in only 25-50% of infected cases (3-5). Detection of the organism by culture is necessary for diagnosing but culture is time consuming or is sometimes negative due to the low numbers of organism. On the other hand, upon primary isolation, definitive identification of unknown yeast as *C. neoformans* requires carbon assimilation and other tests, which extend the period before the clinical report is provided to physician. Therefore, use of a test with high sensitivity and specificity is required for rapid and early diagnosis of the disease. It has been shown that detection of free

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cryptococcal polysaccharide antigen in body fluid, i.e., cerebrospinal fluid (CSF) or serum by latex cryprococcal antigen test (LCAT) is indicative of infection in 98% of central nervous system (CNS) cryptococcosis during a very short time (6). Since at present commercially available kit is purchased expensively from foreign countries, so the present study was undertaken for the first time to develop this rapid serological method for diagnosis of cryptococcosis in Iran, that could be carried out in almost any clinical microbiology laboratory.

Materials and Methods

C.neoformans NCPF 3168 was used throughout the experiments. It was grown on glucose peptone modified agar (GPMA) in petri dishes at 37°C for 42-72 h. The yeast cells were harvested and washed in sterile 0.85% saline by centrifugation. Then packed cells were resuspended in saline to the final concentration of 2×10^4 cells per ml (OD= 0.18).

In the present study, direct, cultural, histopathological examinations and commercially available latex-crypto detection system (Immy, USA) kit were used as gold standard tests for detection of experimental cryptococcsis and for evaluation of efficacy of OPR as well. So two groups of sixty-day-old female BALB/C mice with average weight of 21.3 g were employed; 38 for production of experimental infection (cases) and other 38 as normal controls. Procedures involving animals and their care were conducted in conformity with national policies.

The case mice were inoculated intraperitonealy (IP) with 0.1ml of 2×10^7 cells in sterile saline. Simultaneously, 38 normal mice were inoculated IP with 0.1 ml sterile saline as control. Then the mice were bled after 28-32 d and the sera were separated for use in LACT. The mice were later autopsied and brain, heart, and lung were checked with direct, touch smears, histopathological and cultural examination. Touch smears were stained with Gimsa (G), India ink (II) and also tissue sections were obtained and stained with Gomory methenamine silver stain (GMS) and Mayer's mucicarmine (MM)method (7).

Culture of specimens from brain, heart and lungs were made on Brain Heart infusion agar (BHIA) and incubated at 30° C and 37° C for 3-5 d.

Antiserum production Initially *C.neoformans* (NCPF 3168) was grown on GPMA at 37° C for 48-72 h. The yeasts were washed from agar surface with 2% sterile formol saline and allowed the cell suspension to stand at room temperature for 18 h. Then the suspensions were centrifuged, supernatant discarded and cells resuspended in sterile 0.85% saline. Later the cell suspension was heated in 56° C water bath for 30 min (8, 9). The cell suspension was centrifuged, the supernatant discarded and the yeasts were suspended in 0.85% sterile saline. A loopful of suspension cultured on sabouraud's dextrose agar(S) to check that cells are non-viable. The cell concentration adjusted to 10^9 cells per ml according to haemacytometer count. The suspension stored at 4° C for further use. This stock cell suspension was used to raise cryptococcal antiserum and as the antigen in the agglutination test.

Anticapsular antiserum of *C.neoformans* raised in nine male 4-6 month-old with average weight of 1.5 kg New Zealand white rabbits by the method of Evans (9). Rabbits were inoculated intravenously (IV) with 0.5 ml of $25x10^7$ formalin-killed *C.neogormans* cells daily for 14 d, $25x10^6$ cells were given on the first day and dose gradually increased to 25×10^7 by the tenth day to minimize the risk of anaphylaxis. The animals were rested for seven days and test bled. The agglutination titer was estimated by direct agglutination method as described by Mackenzie (8) and unless the serum showed a titer of 1:256 or greater, then the immunization schedule was repeated.

Preparation of sensitized latex paricales (SLPS) Immunoglobulins (Igs) from the cryptococcal antiserum were obtained by ammonium sulfate fractionation as mentioned by Evans (9). The protein of the globulin solution

was determined using the Bradford method (10) and adjusted to contain 40 mg protein per ml. Serial doubling dilutions of globulin solution were prepared in sterile glycine buffered saline diluent (GBS) pH 8.2 ranged from 1:50-1: 800 (7, 9).

The carboxylate-modified beads 0.9 µm diameter (sigma, prod. No. CLB-9) was used for preparation of SLPS. Also deionized distilled water (DIW) was employed for diluting and washing the latex particles (LPS) throughout (10, 11-13). The stock suspension of LPS in DIW was such that when diluted 1:100 in round cuvette, its OD was 0.3 at 650 nm in colman model 6A spectrophotometer (8). Then equal volumes of LPS (400-µl v/v) suspension were mixed with the different dilutions of globulin and was stirred at room temperature on a rotary shaker to 50 rpm for 2 h and later stored at 4° C for overnight. Later cryptococcal antigen test was performed using positive control serum (7). The highest globulin dilution that gave unequivocal agglutination was recorded. A stock suspension of SLPS was prepared by mixing equal volumes of latex suspension with maximally reactive globulin dilution in GBS diluent for furthers use.

All sera from case and control mice were examined by LCAT as follows: 20µl of each serum sample was placed onto glass ring slide, later 20 µl of SLPS suspension was added to those samples. The contents of each ring was then thoroughly mixed with separate applicator stick and placed on a rotary shaker set to 120 rpm for 3-5 min. Positive and negative controls were run on each test for comparison with test sera. Results were recorded as negative to 4+ scale by reading reactions against a dark background illumination. The sera found to be positive for cryptococcal antigen were later tittered.

We tested 105 human serum and 15 cerebrospinal fluid (CSF) samples. The patient samples were from first specimens submitted to our diagnostic fungal serology laboratory for cryptococcosis (15 sera, 15 CSF), aspergillosis (n=30) or suspected of having other fungal infections (n=30), and healthy subjects (n=30). Almost all came from patients with culturally proven cryptococcal meningitis and invasive aspergillosis. In contrary to diluent used for mice sera in the test carried out with OPR the diluent for specimens of human serum or CSF was GBS solution, pH 8.2 to which 0.1% bovine serum albumin (sigma) was added (GBS-BSA). Because in the absence of added BSA, minimal non-specific agglutination occurred with highly diluted serum and CSF. In addition, titration was performed when samples were found to be positive and gave a 2+or grater reaction. For this purpose serial dilutions of the specimens were made (1:2 through 1:1024 or higher if is still positive).

Latex- cryptococcal antigen test by Immy

The procedure was carried out according to directions furnished by the manufacture. This kit was tested in comparison with the OPR for both sera from case and control mice also serum and CSF from patients suspected of fungal infection.

Results

Direct examination and also tissue sections of brain from all experimentally infected mice (cases) were positive with four staining methods (II, G, GMS, MM) and cryptococci were recovered in culture (Fig. 1, 2, 3). However, all the above-mentioned tests were negative for controls. The LCAT was positive in 100% of serum specimens from case mice and negative in 100% of control mice by use of Immy reagents.

Direct and histopathological examination of heart samples with four staining methods were positive in 7.8%, 7.8%, 18.4%, 18.4%, 18.4%, of cases, respectively (Fig. 4). However, positive cultures obtained from hearts of 28.1% of cases, but not any organism was recovered from culture of heart specimens of controls.

Direct and histopathological examination of lung specimens with four staining methods showed *C.neoformans* in 18.4%, 13.1%, 34.2%,

34.2% of cases, respectively (Fig. 5, 6). The culture of lung samples were positive for *C. neoformans* in 39.4% of cases. Direct, histopathological and culture examination of lung specimens were negative in all controls.

The result of cultures showed that the *C.neoformans* was recovered after 3 d at 30° C and after 5 d at 37° C.

Specificity, sensitivity, positive and negative predictive values and efficacy of 4 staining methods, culture and LCAT in diagnosis of cryptococcal CNS infection were 100%.

Tables 2, 3 show the specificity, sensitivity, positive and negative predictive values of each of staining methods, culture and LCAT in diagnosis of pulmonary and heart cryptococcosis.

Direct agglutination test for different dilutions of rabbit anticryptococcal globulin, which produced in our laboratory, showed that globulin dilution of 1: 400 in GBS solution was highly reactive. Therefore, GBS dilution of 1:400 globulin was coated onto latex particles and was tested in parallel with the Immy reagents for its efficacy in detection of cryptococcal antigen. OPR tested on 38 serum samples of case mice exhibited 2+ to 4+ agllutination in all but one case with titers similar to those obtained using Immy's kit.

All serum from control mice did not show any agglutination using both OPR and Immy's kit.

Sensitivity; specificity , positive and negative predictive values, efficacy and reliability of OPR in detection of mice cryptococcosis were 97.37%, 100%, 100%, 97.43%, 98.68% and 0.99 respectively (Table 1, 2).

On the other hand, when serum and CSF from patients with cryptococcal meningitis tested with OPR and Immy's kit, the OPR demonstrated titers equal or one to two dilutions lower than the Immy's in all cryptococcal infected patients (Table 3).

Table 1: Comparison of different diagnostic methods in experimental lung cryptococcosis

Concept / method	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Efficiency	Reliability
India ink	18.4	100	100	55	59.2	0.56
Gimsa	13.1	100	100	53.5	56.5	0.54
GMS	34.2	100	100	60.3	67.1	0.61
MM	34.2	100	100	60.3	67.1	0.61
Culture	34.9	100	100	62.3	69.7	0.63
Immy LCAT	100	100	100	100	100	1
OPR LCAT	97.37	100	100	97.43	98.68	0.99

GMS: Gomory methenamine silver MM: Mayer's mucicarmine

Table 2: Comparision of different diagnostic methods in experimental heart cryptococcosis

Concept / method	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Efficiency	Reliability
India ink	7.8	100	100	52	53.9	0.53
Gimsa	7.8	100	100	52	53.9	0.53
GMS	18.4	100	100	55	59.2	0.56
MM	18.4	100	100	55	59.2	0.56
Culture	28.9	100	100	58.4	64.4	0.59
Immy LCAT	100	100	100	100	100	1
OPR LCAT	97. 7	100	100	97.4	98.6	0.99

Table 3: Latex slide reactions of serum and cerebrospinal fluid from patients with cryptococcal meningitis

No. patient	Titer of first specimen			
	CSF		Serum	
	Immy	OPR	Immy	OPR
1	1024	512	1024	512
2	128	64	Neg	Neg
3	1024	1024	1024	1024
4	2048	1024	1024	1024
5	128	64	256	128
6	1024	512	1024	256
7	512	512	1024	512
8	1024	512	1024	512
9	512	256	128	32
10	1024	512	1024	512
11	128	32	128	32
12	256	256	256	256
13	512	128	128	64
14	256	256	256	256
15	1024	512	1024	512

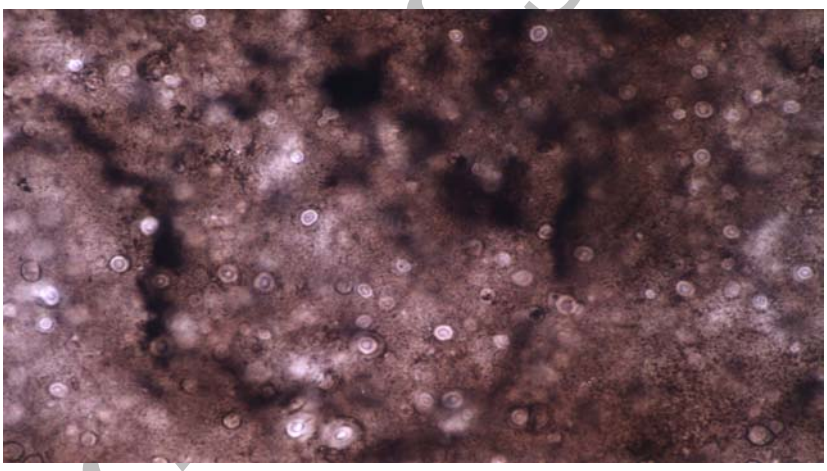

Fig. 1: India ink preparation of brain tissue, showing numerous encapsulated *C. neogormans.* (x 400)

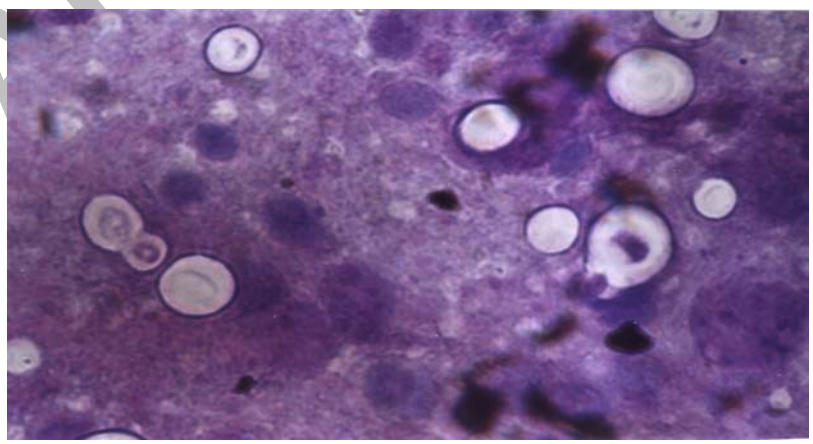

Fig. 2: The encapsulated cryptococcal yeast cells in brain tissue (Gimsa x 1000)

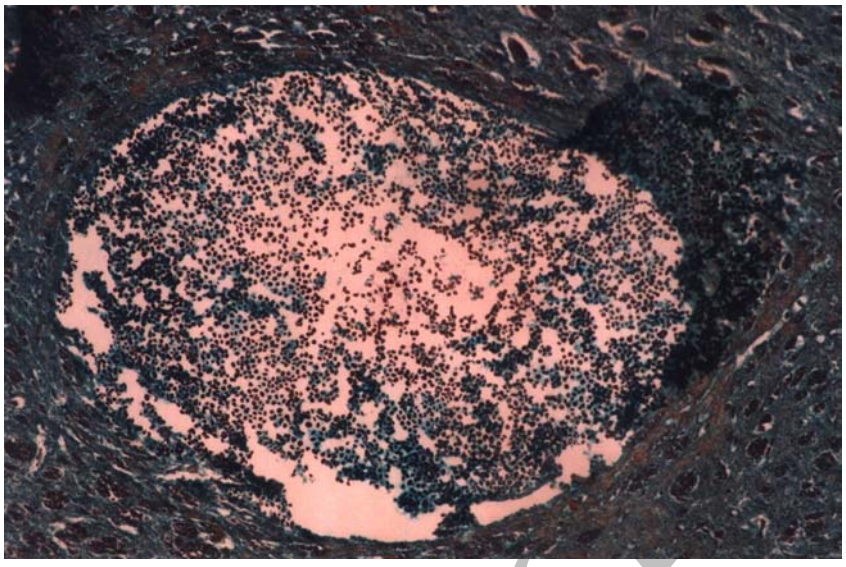

Fig. 3: Histopathological appearance of brain tissue in experimental cryptococcosis, note the micro-abscess containing yeast cells. (GMS x 200)

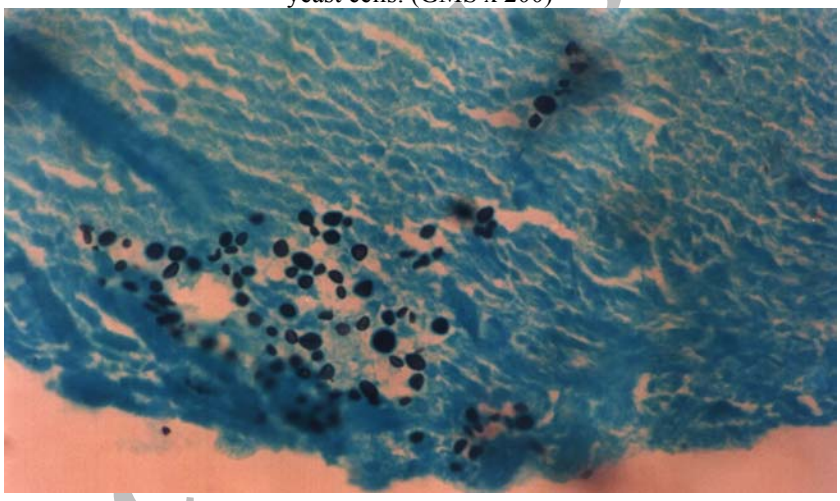

Fig. 4: Histopathological appearance of heart tissue in experimental cryptococcosis, note the micro-abscess containing yeast cells. (GMS x 400)

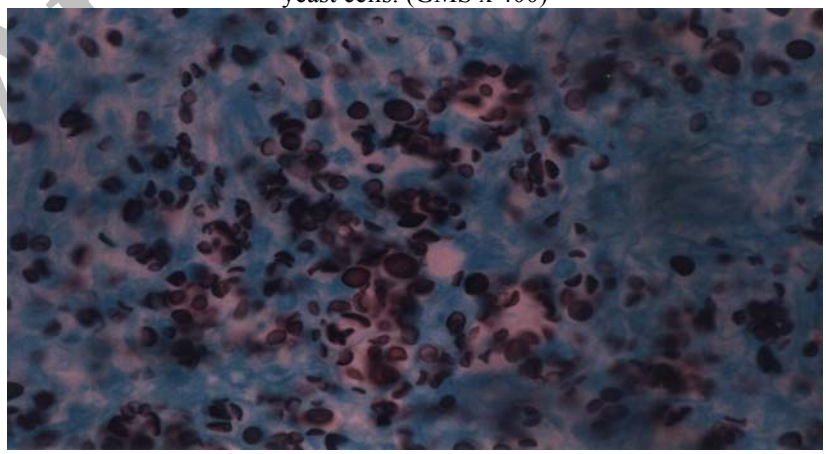

Fig. 5: Histopathological appearance of lung in experimental cryptococcosis. Shown single and budding well stained yeast cells. (GMS x 400)

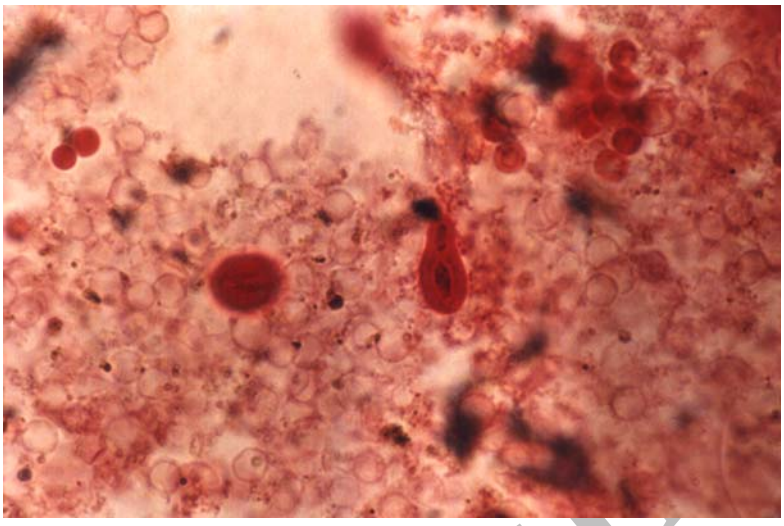

Fig. 6: Histopathological appearance of lung in experimental cryptococcosis, note the mucicarmine-postive capsular material (MM x 1000)

Discussion

Cryptococcosis has become the fourth leading life-threatening opportunistic infection in patients with acquired immune deficiency syndrome (AIDS) (4, 5, 14) but also occurs in non-AIDS patients (12). Recently in view of the increasing number of infections in Iran (1, 2, 15-17), rapid, sensitive and specific diagnosis of cryptococcal disease has become important than ever. The antigen detection test is highly specific and more sensitive compared with microscopic (positive 50%, in non-AIDS and 82% in AIDS patients) and culture (positive 90%, in non-AIDS, 100% in AIDS patients), and it may be the only positive test when used for screening or early diagnosis (18, 19). Diagnostic usefulness of the test was evident particularly in cases where cryptococcosis was indicated in spite of a negative direct microscopic examination of CSF and later demonstration of *C. neoformans* in culture. Furthermore an unequivocally positive test for antigen appears to be reliable enough to warrant therapy. Amphotericin B frequently used in the treatment of systemic mycosis, has a high degree of toxicity. Even though newer, less toxic chemotherapeutic agents may prove useful, but the use of sensitive and specific diagnostic test is believed to be important. Detection of cryptococcal antigens in patient specimen was first described in 1963 (20) and the reliability and usefulness of the test were reported in 1964 and 1966(21, 22). It was also shown that LCAT was rapid and had been documented to be both sensitive and specific (15, 23). Since, the commercial kit of LCAT is purchased expensively from foreign countries; therefore the recent study was undertaken for the first time to develop this rapid serological method for diagnosis of cryptococcosis in Iran that could be carried out in almost any clinical microbiology laboratory.

Although there are currently four commercially available latex crypotococcal antigen tests, but Immy's LACT kit was used in this study beyond to the culture and histopathologic sections as the gold standard in diagnosis of active cryptococcosis. Published studies (15, 24, 25) have given a remarkable sensitivity as high as 97% by Immy's kit when. Our data indicate that the sensitivity and specificity of OPR is quite comparable with the commercial kit in detecting of active cryptococcal infection in both experimentally infected mice and human patients. Most notable between the Immy kit and OPR was the titers of antigen in serum and CSF. By use of OPR, titers of cryptococcal antigen were equal or one or two dilutions lower than Immy kit. We believe that pretreatment of

serum and CSF with pronase in Immy procedure is responsible to this difference. Moreover it has been shown by other investigators that specifically, the use of pronase on serum samples reduces the numbers of false-positive and false-negative results (26, 27). Also in contrast with serum specimens, it dose not appear that pronase treatment is necessary for CSF samples (27). The concurrent use of microscopic, cultural and immunologic procedures enabled us to diagnose 16 cases of cryptococcosis belonging to CNS type from the time this mycosis was first reported in Iran in 1978(28) to the present (15-17). Only 15 had been diagnosed during past decade in Tehran. The LACT (Immy & OPR)demonstrated exceptionally high titer of circulating cryptococcal antigen(256 to 1024). *C. neoformans* infections occurred more commonly in males (nine) than females (six) and there were ten fatal cases of cryptococcosis among those patients. Further, when OPR was controlled with serum from patients with other fungal infections and healthy individuals, not any reactivity was observed. Therefore as a conclusion, the LACT with use of OPR is a quite reliable, reproducible and cost-effective, particularly in countries where the commercial kit is not generally available and must be obtained expensively. Besides, the test also has prognostic value as much as response to treatment was reflected in declining reactivity and unchanged or increased titer of antigen is associated with failure to respond to therapy.

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