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IMMUNO DIAGNOSIS OF ENDOPHTHALMITIS BY DETECTING VITREOUS FLUID ANTIBODY USING ENZYME LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

The novelty of the method developed in the present study was the use of vitreous fluid as specimen for the indirect-ELISA. The indirect-ELISA was evaluated by using *C. albicans* whole cell antigen for the detection of IgG antibody in vitreous fluid of *C. albicans* endophthalmitis and control subjects. All these cases were considered with the confirmation of vitreous fluid culture for its more specificity in nature. The indirect-ELISA was given 94.64% of positivity result for the detection of anti-*C. albicans* antibody(IgG) in vitreous fluid. The test quality of indirect-ELISA was showed the sensitivity 91.44%, specificity 100%, efficiency 92.20%, PPV 100% & NPV 53.19%. The reasonable in its specificity and sensitivity for antibody detection in vitreous fluid than antibody detection in serum, the ELISA method developed here might be useful for diagnosis of endophthalmitis candidiasis.

KEYWORDS: Endophthalmitis, Vitreous fluid, *Candida albicans*, ELISA, Antibody

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INTRODUCTION

Endophthalmitis is a serious intraocular inflammatory disorder affecting the vitreous cavity that can result from exogenous or endogenous spread of infecting organisms into the eye (Mamalis, 2002). Successful management of this condition depends on prompt diagnosis and treatment with appropriate antimicrobial therapy. Many attempts have been made to isolate the pathogen from blood, urine and other body fluid but microbiological investigation of vitreous fluid is thought to be the only method that permits reliable identification of the causative microorganisms (Yosanan et al, 2005). Unfortunately, vitreous fluid cultures have limited sensitivity and are often negative or become positive only after a long delay (De Repentigny et al, 1984 & Jones, 1990). Although PCR is a specific and sensitive method in the diagnosis of endophthalmitis, it has high cost, sophisticated process and may result in false positive reaction in some cases (Ginesu et al, 1998). Much effort has been made to develop reliable tests for rapid diagnosis of Candidal endophthalmitis leading to appropriate therapy. The immunological response to fungal infections in humans is complex and still subject to much debate (Shoham & Levitz, 2005). However, the importance of a robust antibody response to *Candida* infections has also been documented. The detection of antibodies directed against different *Candida* antigens was employed by various scientists. Such antibody can be detected by use enzyme-linked immunosorbant assays (ELISA) for the diagnosis of Candidal endophthalmitis. Candidal Endophthalmitis is an infection of tissues inside the eyeball, generally caused by *C. albicans* (Edwards et al, 1974; Okada et al, 1994; Mehta et al, 2007). From our recent study 52.11% of *C. albicans* was the predominant isolate of mycotic endophthalmitis in Odisha, Eastern India (Patra et al, 2012). The intraocular 'immune-privileged site', devoid the inflammatory mediators and cells present that would otherwise fight against infection (Medawar 1948; Taylor 2009). The ciliary body and iris may act as local factories for the production of antibodies. The analysis of the immunoglobulin (Ig) composition of the vitreous (Nguyen et al, 2001) have suggested that there may be local (intraocular) production of immunoglobulins as an immunologic response in ocular inflammatory diseases. Hence, the current study vitreous fluid analysis is primarily targeted in order to qualify its suitability for a test specimen in terms of antibody detection against *C. albicans* compared to that using peripheral blood.

MATERIALS AND METHODS

Patients and controls

The samples of peripheral blood and vitreous fluid were collected from the local tertiary care Eye Hospital between the study period September-2008 to December-2011. Microbiological investigation of vitreous fluid was done as described earlier (Patra et al, 2012) and the culture report was taken as parameter for confirmation and consideration of positivity/negativity of test result. The following groups of samples were considered for the present study.

Group-I: *C. albicans endophthalmitis patients:* A total 235 endophthalmitis patients were included as confirmed (culture proven) *C. albicans* cases.

Group-II: *Disease controls:* A total 15, other than *C. albicans* endophthalmitis cases (5 Aspergillosis, 5 Fusarium and 5 Staphylococcal) were taken for observation of cross-reactions in the immunological assays.

Group-III: *Normal Healthy control:* A total of 25 healthy individuals without any known clinical evidence of endophthalmitis were included in this study as negative controls.

Specimens

About 0.2 to 0.3 mL of vitreous fluid samples from above study group of 275 cases were preserved at -20^0 C until used. Subsequently 0.5 mL of sera was separated aseptically from same study group of each case and stored at -20^0 C.

Preparation of antigen

Standard reference strain P37005 (Genotype: *MTLa/MTLa;* Wild-type clinical isolate) was used to prepare whole cell antigen as per the method described elsewhere (Nils, 1973). The strain was cultured aerobically for 48 hrs at 35 ± 2^0 C on Sabouraud's dextrose agar (SDA) medium containing 0.05 mg/ml of Chloramphenicol. Colony was gently scraped off from SDA plates with cell scrappers and suspended in sterile saline (0.154 M NaCl). The culture was washed three times in 0.154 M NaCl. First washing was done with pre-weighed 50-ml glass tube by sedimenting at 1,500 rpm for 10 min. The supernatant fluid is discarded, and the sedimented yeast cells were re-suspended in saline (about 1:1, vol/vol). The first step was repeated for second time. The third centrifugation was at 4,000 rpm for 15 min after which the supernatant fluid was discarded. After this washing procedure the wet culture remaining in the tube was collected and the cells were suspended in sterile saline at 1:2 ratio. Approximately two grams of wet culture was suspended with equal volume of saline water and transferred to a 10 ml Braun homogenization bottle containing 10.0 g of glass beads (0.45-0.50 mm). Homogenization was performed in a Braun cell-homogenizor (MSK) at 2,800 rpm for 2 min. by using liquid CO_2 as a coolant. After this procedure controls assured that the bottle was ice-cold, and that the contents were not frozen. The homogenized culture without glass beads was submitted to ultracentrifugation at 4^0 C for 1 hr at105, 000 x g. After the centrifugation there was a turbid surface layer, and above the dense sediment there was another turbid zone. These two layers were avoided in pipetting the clear supernatant fluid which was used as antigen.

Indirect-ELISA for detection of anti-Candidal IgG antibody in serum and vitreous fluid

The indirect-ELISA was evaluated by using *C. albicans* whole cell antigen for the detection of IgG antibody in the serum and vitreous fluid samples from cases of *C. albicans* endophthalmitis and control subjects. The optimum antigen concentration for coating microtiter wells and sample dilution was standardized by checkerboard titration. The concentration of antigen and dilution of sample, which gave the maximum ratio of OD values with known positive serum/vitreous fluid and that with negative serum/vitreous fluid, were considered to be the optimal conditions for the test. All the test serum samples were analyzed at the dilution (1:100) in PBST, whereas the un-diluted vitreous fluid was used for detection of antibody. The indirect-ELISA was done as described earlier (Na and Song, 1999 & Emma et al, 2000) to detect antibodies IgG in serum and vitreous fluid against *C. albicans*. Briefly 1 µg of purified *C. albicans* antigen diluted in carbonate buffer (pH 9.4) per well was coated in 96-well Polystyrene plates for overnight at 4^0 C. After overnight incubation the un-absorbed antigen was removed by washing the plates three times with phosphate-buffered saline containing 0.05% Tween 20 (PBST). The uncoated reactive sites in the wells were blocked by incubating with 200 µl of 1% bovine serum albumin (BSA) in PBST per well for 2 h at 37°C. Plates were washed three times with PBST. *Primary antibody (from patient's specimen):* A total 100-µl volume of sample was added directly to each wells and incubated at 37^0C for 2 hrs. The plates were washed 3 times in PBST as before to remove unbound antibodies in sample. *Secondary antibody (conjugate) incubation:* Goat anti-human IgG horseradish peroxidase-conjugated secondary antibody (Sigma Chemical Co., St. Louis, Mo.) was used as per manufacturer's instruction of 1:3,000 dilutions in PBST. 100 µl of volume was suspended to all the wells and incubated at 37^0 C for 2 hrs. Plates were washed 3 times with PBST as before to remove unbound conjugate. *Plate development:* Substrate solution was prepared freshly by dissolving 0.4 mg of *o*-phenylenediamine (Sigma) per ml in 0.05 M citrate buffer (pH 5.2) and then adding hydrogen peroxide (H_2O_2) at a final concentration of 0.005%. 100 µl of prepared substrate solution was added to each well and incubated for 20 min in darkness. The reaction was stopped by adding 50 µl of 4 N H_2SO_4 per well. The absorbance was recorded at 490 nm (OD_{490}) using an ELISA reader (Microplate

Reader 450; Bio-Rad, Richmond, Calif.). Each test series included a test sample for positive control as well as a negative control, both were analysed under the same conditions. The ELISA procedure was repeated by using *C. albicans* antigen for the testing of IgG in serum and vitreous samples from cases of *C. albicans* endophthalmitis group as mentioned earlier. Each assay was repeated for at least three times and the results were expressed as mean OD for each determination.

Determination of cut-off titre

The cut-off titers were determined by calculating an average OD of 12 normal serum/vitreous fluid at 490 nm (OD_{490}) plus 3X standard deviations. Test results were considered positive if the test sample OD value was exceeded the cut-off value.

Estimation of test quality

In this study the newly improved ELISA for detection of anti-Candidal antibody IgG present in specimen of endophthalmitis was evaluated for the establishment on sensitivity, specificity, efficiency and predictive value of the test result.

RESULTS

In the indirect-ELISA, for the detection of anti-*C. albicans* antibody(IgG) from serum and vitreous fluid, the cut-off value were summarized by the table-1(Table-1). The cut-off OD_{490} value for detection of antibody IgG in serum was estimated to be 0.163, whereas, the cut-off OD_{490} value for detection of antibody IgG in vitreous fluid was estimated to be 0.172 by using *C. albicans* whole cell antigen.

Table-1
Estimation of the cut-off OD_{490} values for the indirect-ELISA for detection of IgG in Serum and Vitreous fluid

OD_{490} normal sample	Estimation of cut-off value in different specimen by using indirect-ELISA	
	Blood (n=12)	**Vitreous fluid (n=12)**
OD range	0.088-0.137	0.098-0.152
Mean OD	0.115583	0.12475
SD	0.015848	0.015575
Cut-off	**0.163**	**0.172**

[SD: Standard deviation; OD: Optical density; n: number of test samples]

The optical density (OD_{490}) range was found to be, 0.058 to 0.730 for serum analysis. OD values of a total 158(67.23%) sera were above the cut-off titre and the OD values of 77 (32.77%) sera were less than the cut-off titre. Based on the cut-off value, the ELISA test was given 67.23% of positive and 32.77% of negative results out of 235 microbial confirmed *C. albicans* positive samples. The result of *C. albicans* endophthalmitis and controls were summarized in table-2 (Table-2). In this present study for analysis of vitreous fluid the optical density (OD_{490}) range was found to be, 0.077 to 0.811, whereas, the indirect-ELISA test was given 213(94.64%) of positive and 22(9.36%) of negative results out of 235 microbiological confirmed *C. albicans* endophthalmitis vitreous samples.

Table-2
Detection of IgG antibody in serum and vitreous fluid by Indirect-ELISA

Study group	No. of subjects	TEST RESULT OF SERUM		TEST RESULT OF VITREOUS FLUID	
		Positive (%)	**Negatives (%)**	**Positive (%)**	**Negatives (%)**
I	235	158(67.23)	77(32.77)	213(94.64)	22(9.36)
II	15	4(26.67)	11(73.33)	2(13.33)	13(86.67)
III	25	4(16.00)	21(84.00)	0	25(100)

[Group-I: cases of C. albicans endophthalmitis; Group-II: Disease control (other than C. albicans endophthalmitis); Group-III: healthy controls]

Out of 15 disease control serum samples 4 cases (*Aspergillus sps*: 1, *Fusarium sps*: 1, *Staphylococcal sps*: 2) of non-candidal endophthalmitis were showed to be positive by indirect-ELISA, using *C. albicans* antigen. From 25 cases of normal negative control sera, 4 cases were showed to be positive by the indirect-ELISA test. In case of vitreous fluid analysis, all the 25 negative control samples were given purely negative result by indirect-ELISA test, whereas, from 15 disease controls of non-candidal endophthalmitis cases, a total 2(13.33%) cases were found to be positive. It included one strain of *Aspergillous* and one strain of *Fusarium*. The test quality of the indirect-ELISA for both serum and vitreous fluid was summarized in table-3 (Table-3). The sensitivity of the test for detection of anti-Candidal antibody IgG in serum was found to be 75.32% and the specificity was 86.21%. The efficiency of the test system was 76.25%, whereas, positive predictive value (PPV) and negative predictive value (NPV) were 98.33% and 24.51% respectively. The test quality of indirect-ELISA for detection of anti-Candidal antibody IgG in vitreous fluid was showed 91.44% of sensitivity and 100% of specificity. Efficiency of this test system was found to be 92.20% whereas the PPV & NPV were found to be 100% & 53.19% respectively.

Table-3
Test quality calculation for detection of antibody IgG by indirect-ELISA

Test quality parameters	Frequency (%) of reactivity in different specimen	
	Blood(%)	Vitreous fluid(%)
Sensitivity(%)	75.32%	91.44%
Specificity(%)	86.21%	100%
Efficiency(%)	76.25%	92.20%
PPV(%)	98.33%	100%
NPV(%)	24.51%	53.19%

[Sensitivity: TP/(TP+FN); specificity: TN/(FP+TN); positive predictive value: TP/(TP+FP); negative predictive value: TN/(TN+FN); efficiency: (TP+TN)/(TP+FP+TN+FN) (where TP: true positive, FP: false positive, TN: true negative, and FN: false negative]

DISCUSSION

There have been several head-to-head comparisons of the various assays for detection of the *Candida* antigens. Unfortunately, none of these assays have performed well enough or has a good enough predictive value at this point to be able to recommend its routine use in a clinical laboratory (Pasqualotto and Denning, 2005). During the last decades, the use of antibody tests for the diagnosis of invasive mycoses has declined as an onsequence of the general belief that they are insensitive and non-specific. However, there is clear evidence that antibodies can be detected in highly immunodeficient patients, and those antibodies are useful for the diagnosis (Quindos et al, 2001). Although in most studies anti-*Candida* IgG antibodies were detected in serum, other specimen can also investigated in some studies. Some workers have analyzed the vitreous fluid of uveitis cases (Baarsma et al, 1991; Nguyen et al, 2001) for detection of antibody. Endophthalmitis results in inflammation of all eye tissue including vitreous and anterior chamber. Infectious organisms can stimulate the cells for spontaneous secretion of IgG or IgM antibodies in the common environment (Dar O et al, 1990). Moreover, high titers of antibody may be the results of simple colonization of *Candida sps.* (De Repentigny et al, 1994). Specific antibodies in the vitreous fluid may be suggestive of an infection. It suggests that IgG may be detected in vitreous fluid of *C. albicans* endophthalmitis patients. Therefore, a method was developed for diagnosis of *C. albicans* endophthalmitis by means of detection of local antibody in vitreous fluid. Many others scientists have also suggested to the possible diagnostic use of this specimen (Baarsma et al, 1991; Nguyen et al, 2001; Mathis et al,1988). However, a larger prospective study with comparison of different specimen (Blood and vitreous fluid) for diagnosis of candidal endophthalmitis is

needed to generalize this method for clinical use. The results obtained from the two different specimen using indirect-ELISA procedures are summarized in Table-2 & Table-3. The serum sample showed 67.23% of positive and 32.77% of negative results whereas, vitreous fluid showed 94.64% of positive and 9.36% of negative results. For the detection of anti-Candidal antibody IgG in serum, the sensitivity, specificity, efficiency, PPV, NPV were found to be 75.32%, 86.21%, 76.25%, 98.33% & 24.51% respectively. Whereas, the detection of antibody IgG in vitreous fluid, the sensitivity specificity, efficiency, PPV, NPV were found to be 91.44%, 100%, 92.20%, 100% & 53.19% respectively. As expected, the vitreous fluid for the detection of antibody by indirect-ELISA showed a relatively high sensitivity, 91.44%, and a specificity of 100%. Other workers have showed different result by using different-ELISA methods. The sensitivity and specificity values of Na & Song (1999) were 93.9% and 96.0%, respectively, for the inhibition-ELISA by using SAP1 antigen. Jiang et al. (1990) have reported in serum and CFS with sensitivities of 96.1% and 100% respectively for Dot-ELISA. In my study, the values for all categories which are meaningful for determining the usefulness of diagnostic procedures were improved significantly. The serum sample obtained from non-Candidal endophthalmitis patients (Aspergillus, Fusarium and Staphylococcal endophthalmitis) was showed 26.67% of positive reactions against anti-*C. albicans* antibody, whereas, the vitreous fluid obtained from above cases was showed 13.33% positive result. By ELISA there may be cross-reactivity among antigens of closely related fungi species (Shen et al, 1990; Na and Song, 1999). *Aspergillus sps*. secretes aspartic proteinase that gives positive result (Inoue et al, 1991; Reichard et al, 1995) against anti-*C.albicans* antibody. Other sps (Fusarium and Staphylococcus) were probably resulted from the antibody stimulation of the immune system and reactivity of antigen with that non-candidal antibody (Portnoy et al, 1987). Those results led to believed that either patients had previously the anti-*C. albicans* antibody or different antibodies were showed cross-reactivity against *C. albicans* antigen. The current study demonstrated that the potential value of antibodies in the laboratory assessment of patients with candidal endophthalmitis. However, IgG antibody analysis from vitreous fluid confirms only the *C. albicans* endophthalmitis, not the species involved in infection. No consistent difference was found in antibody responses to the *C. albicans* by indirect-ELISA. The results suggested that these methods for detection of antibody IgG in the vitreous fluid by using *C. albicans* antigen for endophthalmitis candidiasis is more reasonable in their specificity and sensitivity than antibody detection in serum sample. Therefore, the ELISA method developed here might be useful for diagnosis of endophthalmitis candidiasis.

CONCLUSION

In this study the detection of anti-*C. albicans* antibody in vitreous fluid by indirect-ELISA was found to be an effective result rather than the antibody detection in serum. However, for development of a test system for diagnosis of candidal endophthalmitis, further studies are required to evaluate its effect on other pathogens. At the same time the cross-reactivity with other antibody should be observed.

REFERENCES

1. Mamalis N. Endophthalmitis. J Cataract Refract Surgery, 28(5): 729-730, (2002)
2. Yosanan Yospaiboon, MD,Sarawuth Saree, MD and Sirichai Pasadhika, MD. Blood Culture and Conventional Media for Vitreous Culture in Infectious Endophthalmitis. J Med Assoc Thai, 88(5): 639-42, (2005)
3. De Repentigny L. and Reiss E. Current trends in immunodiagnosis of candidiasis and aspergillosis. Rev Infect Dis, 6:301–312, (1984)

4. Jones, J.M. Laboratory diagnosis of invasive candidiasis. Clin. Microbiol. Rev, 3:32-45, (1990)
5. Ginesu F., Pirina P., Sechi L.A., Molicotti P., Santoru L and Porcu L. Microbiological diagnosis of tuberculosis: a comparison of old and new methods. J Chemother, 10: 295-300, (1998)
6. Shoham, S. & Levitz, S.M. The immune response to fungal infections. Br J Haematol, 129: 569–582, (2005)
7. Edwards J.E. Jr, Foos R.Y., Montgomerie J.Z. and Guze L.B. Ocular manifestations of candida septicaemia: Review of seventy-six cases of hematogenous candida endophthalmitis. Medicine, 53: 47-75, (1974)
8. Okada AA, Johnson RP, Liles WC, D'Amico DJ, Baker AS. Endogenous bacterial endophthalmitis, report of a 10-year retrospective study. *Ophthalmology,* 101: 832-838, (1994)
9. Mehta S, Jiandani P and M Desai. Ocular Lesions in Disseminated Candidiasis. JAPI, 55: 1-3, (2007)
10. Patra GC, Panda P, Sahoo S, Ranasingh R and Nanda PK. Aetiology of Mycotic Endophthalmitis in Orissa, Eastern India. Enviornment Ecology, 30 (1): 159-162, (2012)
11. Medawar P. Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain to subcutaneous tissue, and to the anterior chamber of the eye. *Br. J. Exp. Pathol,* 29: 58-69, (1948)
12. Taylor AW. Ocular immune privilege. *Eye*, 23: 1885-1889, (2009)
13. Nguyen QD, Humphrey RL, Dunn JP, Humayun MS. Elevated vitreous concentration of monoclonal immunoglobulin manifesting as schlieren in juvenile rheumatoid arthritis-associated uveitis. Arch Ophthalmol, 119(2):293-6 (2001)
14. Patra G.C., Sahu PS, Panda P, Sahoo S, Mohapatra S & Bindhani BK. Anti-Mycotic Effect of 'KUSUM OIL' Extract On *Candida albicans*clinical isolates from endophthalmitis cases, 3(2): 475-484, (2012)
15. Nils Holger Axelsen.Quantitative Immunoelectrophoretic Methods as Tools for a Polyvalent Approach to Standardization in the Immunochemistry of Candida albicans. Infection and Immunity, 7(6):949-960, (1973)
16. Na B and Song C. Use of Monoclonal Antibody in Diagnosis of Candidiasis Caused by *Candida albicans*: Detection of Circulating Aspartyl Proteinase Antigen. Clin Diagn Lab Immunol, 6(6): 924–929, (1999)
17. Emma E.M. Jaeger, Nora M. Carroll, Sarah Choudhury, Anthony A.S. Dunlop, Hamish M.A. Towler, Melville M. Matheson, Peter Adamson, Narciss Okhravi and Susan Lightman. Rapid Detection and Identification of *Candida*, *Aspergillus*, and *Fusarium* Species in Ocular Samples Using Nested PCR. Journal of Clinical Microbiology, 38 (8): 2902-2908, (2000)
18. Pasqualotto A.C and Denning D.W. Diagnosis of Invasive Fungal Infections–Current Limitations of Classical and New Diagnostic Methods. Business briefing: European oncology, 1-11, (2005)
19. Quindos G, Moragues MD & Ponton J. Is there a role for antibody testing in the diagnosis of invasive candidiasis? Eur J Clin Microbiol Infect Dis, 20:864-870, (2001)
20. Baarsma G.S., Luyendijk L., Kijlstra A., De Vries J., Peperkamp E., Mertens D.A. and Van Meurs J.C. Analysis of local antibody production in the vitreous humor of patients with severe uveitis. American Journal of Ophthalmology, 112(2): 147-150, (1991)
21. Dar O., Salaman M.R., Seifert M.H. and Isenberg DA. Spontaneous antibody-secreting cells against DNA and common environmental antigens in systemic lupus erythematosus, 3 (5):523-30, (1990)
22. De Repentigny, Kaufman L., Cole G.T., Kruse D., Latge J.P. and Matthews RC. Immunodiagnosis of invasive fungal infections. J. Med. Vet. Mycol. 32(Suppl.1): 239-252, (1994)
23. Mathis A,F. Malecaze, M.H. Bessieres, J.L. Arne, J.P. Seguela and P. Bec. Immunological analysis of the aqueous

humour in candida endophthalmitis. II: Clinical study. Br J Ophthalmol, 72:313-316, (1988)
24. Jiang L., Shen, Y.P. and Zhao W.X. Studies on specific serological antigens in metacercariae and juveniles of Paragonium westermani and its monoclonal antibodies. Sci. China B, 33(2): 178-187, (1990)
25. Shen HD. Cross-reactivity among antigens of different air-borne fungi detected by ELISA using five monoclonal antibodies against Penicillium notatum; Zhonghua Yi Xue Za Zhi (Taipei), 46(4), 195 – 201, (1990)
26. Inoue H., Kimura T., Makabe O. and Takahashi K. The gene and deduced protein sequences of the zymogen of *Aspergillus niger* acid proteinase. A. J Biol Chem, 266:19484–19489, (1991)
27. Reichard U, Eiffert H, Rüchel R. Purification and characterization of an extracellular aspartic proteinase from *Aspergillus fumigatus*. J Med Mycol, 32:427–436, (1995)
28. Portnoy J, Chapman J, Burge H. Epicoccum allergy: skin reaction patterns and spore mycelium disparities recognized by IgG and IgE ELISA inhibition. Ann Allergy, 59: 39–43, (1987).