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Coagglutination Test for Rapid diagnosis of Cryptococcosis: Development and validation

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Abstract

Cryptococcus neoformans is a major opportunistic fugal pathogen and is also an etilogical agent of Aids defining illness causing meningitis.Other immuno compromising condition like cancer & chemotherapy, diabetties predispose the onset of this infection. Many diagnostic methods are available. Among them in our country, direct detection from clinical sample, culture and serological tests like antigen detection tests are practiced. Direct demonstration by culture lack sensitivity &time consuming.Latex agglutination tests done with clinical samples, but the reagent is costly and has to import. Co agglutination test is easy to perform, reagents can be prepared in house in tertiary care centre labs & will incur very minimum costs and result is reproducible.

Keywords: NIL

Introduction

Aims & Objectives:

1. Production & purification of polyclonal antibody against Cryptococcus neoformans var neoformans.
2. Optimization of coagglutination assay using this antibody.
3. In vitro validation of the assay system in comparison with latex agglutination assay.

Materials and Methods

Standard strain & subculture: ATCC 34871 strain of cyptococcus neoformans has been subcultured on sabourands Dextrose Agar(SDA) plate. Gram stain is done for purity check from single colony.

Clinical samples and isolates: Clinical samples like Serum, CSF, and Urine were collected from suspected and confirmed cases of Cryptococcosis. They were stored at (-20^{0}C) till further evaluation.

Antigen: Preparation of whole killed cell antigen (wkca) of C.neoformans was prepared following the protocol of Wilson[1](Wilson et al 1968). Briefly cells were harvested in physiological saline & again suspended in 0.5% formalin & incubated at 4^{0}c.The formalin was removed by repeated washing with normal saline. Cells counted using a haemocytometer. Aliquots of approx 2 X 10^8 cells were stored at (-20^{0}c).This was used for immunisation of rabbit to generate polyclonal antibody and for determining the antibody titre by slide agglutination.

Antibody Generation: Male white rabbits approximately 1.5kgs reared at central animal facility of AIIMS were for used for raising immune serum.

Immunisation Procedure: Immunisation protocol of Dolan[2] (Dolan1977) was followed .2 X 10 ^{8} cells/ml of saline were injected intravenously for five consecutive days with a gap of 2 days/week for total of three weeks and fifteen injections. Trial after last

injection revealed low antibody titre. Two boosters were given on every fourth day. After last booster satisfactory antibody titre could be demonstrated by slide agglutination. Finally the animal was exsanguinated, sera separated and stored at (-20^{0}c) for further use.

This antibody was used for coating the Staphylococcus aureus Cowan -1 strain for development of co-agglutination test.

Slide agglutination: This was carried out for presence of specific antibody in sera using 2x10^8 cells /ml of WKC· Slide agglutination done with neat & doubling dilutions of anti sera mixing with 50µl of WKCA. Antibody titre was determined.

Purification of polyclonal antisera: Was done by precipitation of antiserum using 40% of ammonium sulfate as described by Arunan[3] (Arunan, 1992). The collected pellat was dissolved in 5ml PBS and dialysed extensively with 3 changes against PBS at 4^{0}c to remove ammonium sulphate. The pellat was aliquoted with 1ml PBS. Protein content measured using UV spectrophotometry (uv-160A). Aliaquotes stored at (-70^{0}c) with 0.02% sodium azide[4]. (Stoschek 1990). Protein concentration calculated by the formula (Abs 280x1.58)-(ABS260x0.76).

Preparation of Staphylococcus aureus Cowan -1, Strain for coagglutination reagent: A blood agar plate was streaked with a single colony of Staphylococcus aureus Cowan -1 strain. A single colony was used to inoculate a 50ml culture of Penassay media, incubated overnight to saturation with aeration. This 50 ml culture was used to inoculated 50 ml culture of Penassay media.Growth was allowed to saturation with aeration.The bacterial growth was harvested by centrifumigation in centrifuge tubes at 10,000rpm. The supernatant was discarded & the pellat resuspended in 50 ml PBS with 0.02% sodium azide. The bacteria were again resuspended to 10% wt/vol in PBS with 0.02% sodium azide.

To the suspension while stirring formaldehyde was slowly added up to a final concentration of 1.5%. The mixture was stirred for 90 min at room temperature. The cells were again centrifuges and the pellat was resuspended at 10% wt/vol in PBS with 0.02% sodium azide. This suspension was placed in a 80^{0}C water bath for 5 min with continuous stirring. It was transferred to an icc bath for cooling, Again this suspension was centrifuged as before and resuspended as a 10% vol/vol suspension[5] (Harlow & Lame, 1988).

Antibody Coating: Antibody was coated on Staphylococci by mixing 1 ml of staphylococcal suspension with 1 ml of antisera. Separate reagents were prepared with purified and unpurified antisera. Coagglutination reagents were stored at 4^0 c with sodium azide (0.02%).

Preparation of antigen containing extracts(Test & control): Cryptococcus neoformans was initially cultured on SDA & then subcultured over 72 hrs. Antigen containing extracts (CNPSA) were prepared by suspending the growth in PBS as described by Facklam[6] (1980) from Cryptococcus neoformans.

1. Antigen from following organisms was also prepared by nitrous acid extraction method:
2. Klebsiella pneumonia
3. Haemophilus influenza
4. Srteptococcus pneumonia.
5. Trichosporan beigelii

The above mentioned preparations were used to check the specificity of polyclonal antibodies. The antigen extracted by nitrous acid extraction method following the protocol of Facklam[6].

Carbohydrate content of the extracted polysaccharide was estimated by phenol sulphuric acid protocol (Ashwell, 1996)^0.

Coagglutination Procedure: Using checker board analysis, standardisation of the assay was performed by recording the agglutination at different dilution of the CNPSA with different concentration of the coated Cowan -1 strain of Staph aureus. The results were compared with latex agglutination.

Standardisation of Co-agglutination using extracted cryptococcal polysaccharide antigen was done and the results are shown in table -1: Dilution of CNPSA were prepared from neat (4000ng/20µl) to 1:80(50ng/20µl). These were reacted with dilutions of coagglutination reagent from neat to 1:16 in a checker board pattern to determine the sensitivity and optimal dilution of coagglutination reagent.

Validation on clinical sample: From June 1999 to April 2001, a total of fourteen urine samples (of post

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therapy) from culture confirmed cryptococcal meningitis/ Pneumonitis/ fungaemia cases were taken for validation of the coagglutination test(Table-3). The details of clinical spectrum of infection of these patients are recorded (Fig:3&4).

Observation and Results: The titre of the antibody obtained by slide agglutination test was 40.Standardization of coagglutination reagent by checker board titration, shown in table-1. The test was able to detect up to 50ng of CNPS (measured in terms of galactose concentration in CNPSA). Latex agglutination could also detect 50ng of this antigen i.e sensitivity of the two tests were similar. The coagglutination reagent worked upto a dilution of 1:4, through best results were obtained with neat reagent.

Clinical spectrum from 14 culture confirmed cases of Cryptococcosis: The diagnosis of these cases was confirmed by either direct demonstration and or culture in the specimen like CSF, Sputum, and Blood. Interestingly in about 21 %(approx) cases, no apparent factor could be ascertained. Major associated infection is HIV/AIDS along with Tuberculosis.

Sensitivity of indigenous coagglutination test and commercial latex agglutination test for clinical samples: The results of COA& its comparison with LA in urine samples from 14 culture confirmed Cryptococcal Meningitis/Pneumonitis/fungaemia cases are shown in table-3; of these 14 cases direct Indian ink preparation of CSF was positive in 92.8% cases. All the 14 urine samples were positive for CNPSA by COA as well as LA. Thus the sensitivity of the COA was 100% in comparison to both LA and culture positivity in other specimen (CSF/Sputum/Blood) of the same patients.

Table-1

CNPSA conc.	Dilution of coated polyclonal antibody				
	Neat	**1:2**	**1:4**	**1:8**	**1:16**
Neat (4000ng)	+++	++	+	-	-
1:10 (400ng)	++	+	-	-	-
1:20 (200ng)	+	-	-	-	-
1:40 (100ng)	+	-	-	-	-
1:80 (50ng)	+	-	-	-	-

+++: Strong agglutination

++: Moderate agglutination

+: Weak agglutination

Table :2

Different antigen	Coagglutination reagent
Klebsiella pneumonia	-
Haemophilus influenza	-
Streptococcus pneumonia	-
Trichosporon beigelii	-
C.neoformans	+

- : No agglutination.

+ : Agglutination

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Table-3

Sl. No	Age /Sex	Associated Infection	Nature of specimen	India Ink(CSF/ Sputum/Blood)	Culture Report (CSF/Sputum)	Co-agglutination Test	Latex test
1	54/M	HIV	Urine	+	+	Positive ++	Positive +++
2	38/M	HIV & Pulmonary Tuberculosis	Urine	+/+	+	++	+++
3	7/M	HIV	Urine	+/+	+	++	++
4	26/M	HIV	Urine	+	+	++	++
5	25/M	HIV	Urine	+	+	++	++
6	35/M	HIV & Pulmonary Tuberculosis	Urine	+	+	++	+++
7	31/M	HIV & Pulmonary Tuberculosis	Urine	+	+	++	++
8	60/F	No apparent associated infection	Urine	+	+	++	++
9	36/F	HIV	Urine	+	+	++	++
10	25/F	No apparent associated infection	Urine	+	+	++	+++
11	31/M	HIV	Urine	+	+	++	++
12	68/M						

* Pulmonary Cryptococcosis.

Beef extract	Yeast extract	Peptone	Glucose	NaCl	K_2HPO_4	KH_2PO_4
0.15%	0.15%	0.5%	0.1%	0.35%	0.37%	0.13%

LA-Latex agglutination, COA – Coagglutination test.

Summary and Conclusion:

1. Dolan's method of rabbit immunisation provided a reproducible schedule for generation of polyclonal antibody, though there could be batch variation in animals, which may not give antibody response as indicated by the response of our first attempt.
2. 40% ammonium sulphate precipitation is an easy and relatively rapid method for purification of polyclonal antibody.
3. However purified antibody had a lowered titre and not good enough for coating with Staphylococcus aureus Cowan -1 strain for preparing coagglutination reagent.
4. The coagglutination reagent was prepared with purified antibody.

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5. No cross reaction was observed with extracted bacterial or yeast polysaccharide antigens, indicating suitably specificity.
6. With extracted CNPSA as low as 50ng of polysaccharide antigen (galactose component) could be detected by the coagglutination test.
7. Evaluation of two tests was carried out in 14 clinical samples by detecting the presence of CNPSA in urine from patients with proven cryptococcal meningitis/ pneumonitis/ fungaemia. Polysaccharide antigen gets concentrated in urine and this provides a good non- invasive specimen for study.
8. Of the 14 clinical cases studied by us, 13 presented with cryptococcal meningitis/ Fungaemia and 1 with pneumonitis.
9. Eight of the 14 cases (57%) were associated with HIV infection alone and another 22% dual infection with HIV and Mycobacterium tuberculosis. No apparent predisposing case was seen in the rest (21%).
10. During evaluation of clinical samples, culture was takes as a gold standard. The sensitivity of coagglutination test matched that of culture and 100% of CSF/Sputum/ Stool culture positive individuals showed CNPSA in their urine.
11. Sensitivity was comparable to those of IMMY latex-Crypto Antigen detection system, which also detected 100% of cases.
12. This system can be used for routine non invasive surveillance of HIV positive individuals for cryptococcal infection.

Therefore keeping in all these issues in mind, this assay has a great potential for use in third world countries where diagnosis at low cost is the need of hour, due to fund constraints. Although the LA tests are rapid, but still now they how to be imported which means spending of foreign exchange.

Our study is an indigenous endeavour to develop a case effective method of disease diagnosis, thereby hoping to help patient management in terms of life expectancy and better quality of life.

Appendix & Observation:

1.SDA(Saboroud dextrose agar 4%) as per standard test book.

2. PBS (Phosphate buffered saline PBS-PH-7-2) as per standard book.

3. CNPSA (Cryptococcal neoformans polysaccharide antigen) reagents,

a. 2N sodium nitrate –(13gm in /litr double distilled water)

b. l0N NaOH (40 gm in 1ltr double distilled water)

c. Acid Acetone (10ml conc HCL in 1 L acetone)

4.4N H_2SO_4

1ml of concentrated sulphuric acid to 8ml of double distilled water.

5. Composition of penassay media: components to be dissolved in double distilled water,

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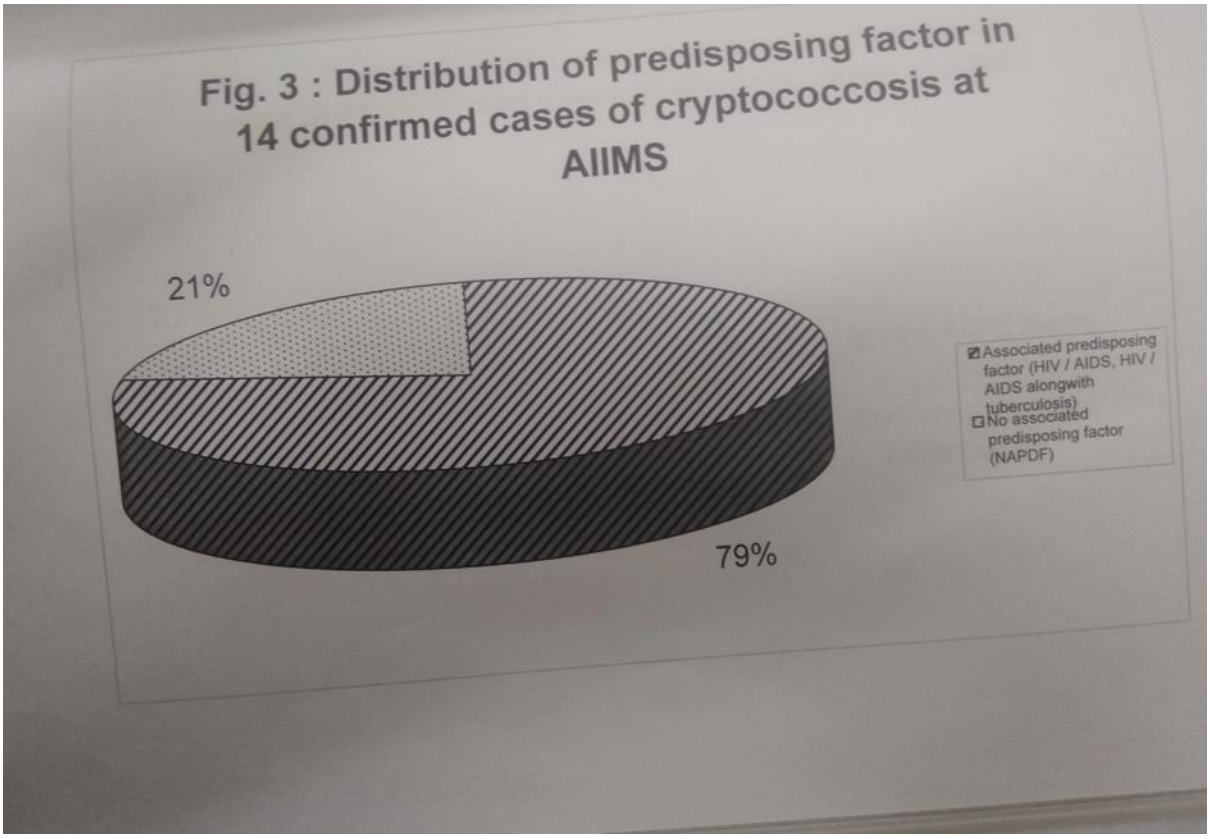

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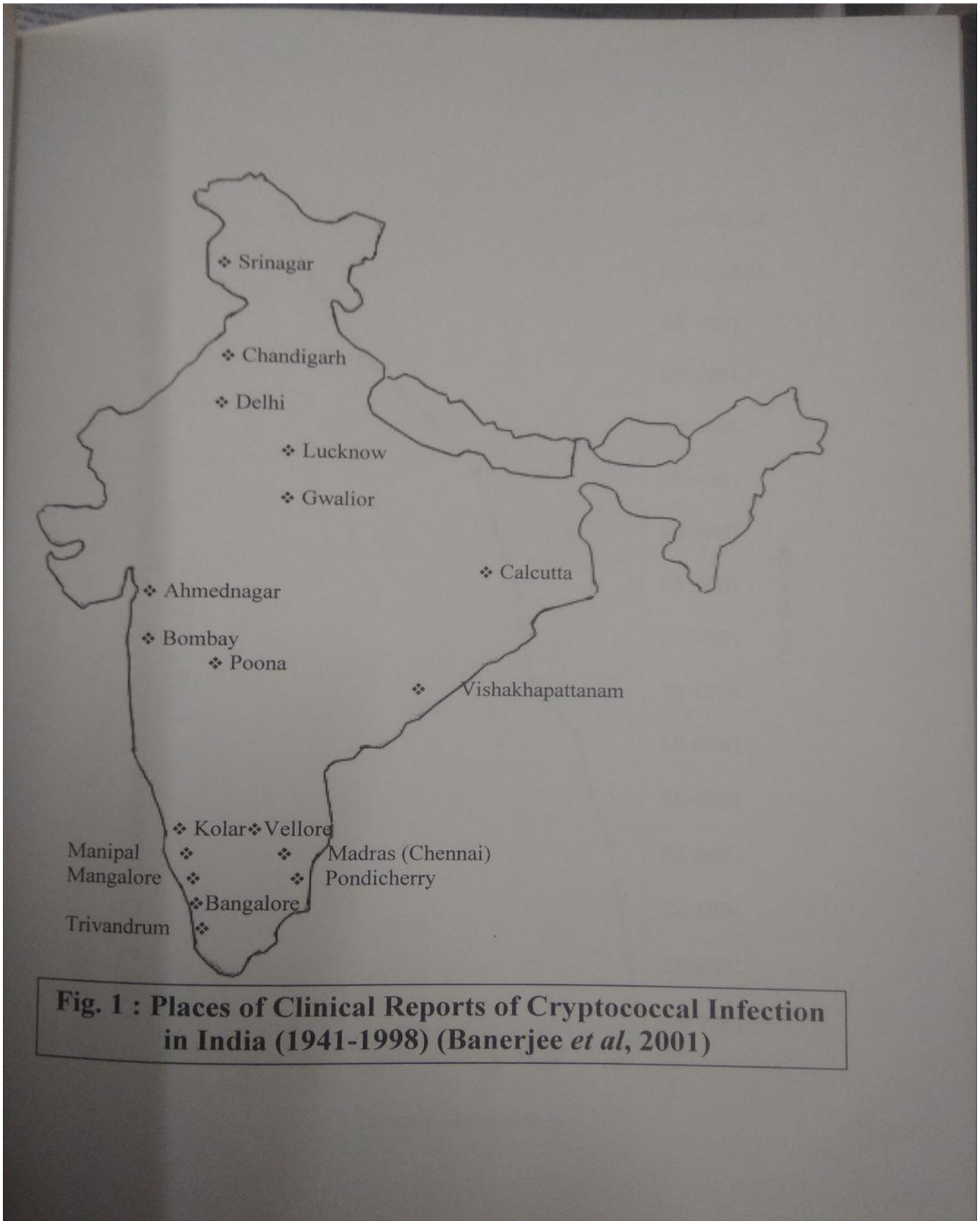

Fig. 1 : Places of Clinical Reports of Cryptococcal Infection in India (1941-1998) (Banerjee *et al*, 2001)

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Fig. 4 : Distribution of immunocompromised status of patient in 11 confirmed cases of cryptococcosis at AIIMS

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