South East Asia Journal Of Public Health
ISSN: 2220-9476
ISSN: 2313-531X (Online)



# Role of Polymerase Chain reaction (PCR) amplifying conserved 5.8S and ITS regions of the cryptococcal DNA in the diagnosis of Cryptococcal meningitis

*Sujata Dharmshale*[1], *Renu Bharadwaj*[2]

[1]Assistant Professor, Department of Microbiology B. J. Govt. Medical College and Sassoon General Hospital, Pune, Maharashtra State, India. [2]Professor and Head, Department of Microbiology, B. J. Govt. Medical College and Sassoon General Hospital, Pune 411001, India.

## Abstract

*Cryptococcus neoformans*, a common fungal pathogen of the central nervous system, results in high morbidity and mortality, unless diagnosed early and specific treatment instituted. The efficacy of the currently available tests for diagnosis (i.e. microscopy and latex agglutination test) are limited. The purpose of the present study was to evaluate a polymerase chain reaction (PCR) test for the diagnosis of cryptococcal meningitis. This cross-sectional prospective study was carried out in a large tertiary care hospital, Pune, India during April 2009 to February 2012. A total of 111 cerebrospinal fluid (CSF) samples were collected from patients with suspected cryptococcal meningitis. All samples were processed for microscopy, culture, antigen detection by Cryptococcal Antigen Latex Agglutination System (CALAS) and PCR using specific primers CN4/CN5. The PCR was evaluated using culture as the gold standard and results compared with those obtained by microscopy and latex agglutination. In the present study 55(49.54%) had laboratory confirmed cryptococcal meningitis (either smear/CALAS/culture/PCR positive). The sensitivity of PCR, antigen detection test and microscopy was 100%, 89.19% and 78.4% respectively while the specificity of these tests was 82.43%, 85.14% & 90% respectively. The positive predictive value of the PCR was 74% and the negative predictive value was 100%. The PCR technique proved to be a rapid and reliable technique for the early diagnosis of cryptococcal meningitis.

**Keywords:** Cryptococcal meningitis, diagnosis, conventional, serological, PCR.

## Introduction

*Cryptococcus neoformans* is a fungal pathogen causing meningitis. This fungal meningitis is more commonly seen in immunocompromised patients; however it has also been reported in immune-competent patients.[1] The route of infection is usually through inhalation and the primary site of infection is the lung.[2] However pulmonary cryptococcosis is a self limiting infection.[2] The organism disseminates haematogenously and has a propensity to reside in the central nervous system (CNS).[3] Patients of cryptococcal meningitis present with moderate to severe headache, fever, malaise and altered sensorium for few days to several weeks.[1] If not diagnosed, may be inappropriately treated, resulting in many complications and a high mortality.[1] Mortality has been reported >26 % from Southern and Northern part of India.[4,5] A recent study from Chiang also reported 52.4% mortality in HIV infected patients.[6]

The currently used diagnostic techniques are demonstration of the pathogen by microscopy, antigen detection and culture.[2] Techniques like microscopy and culture are more specific but less sensitive. Thus other disadvantages are as i) requires large amount of CSF and ii) time consuming.[7] All these techniques have their limitations. Therefore, the present study was undertaken

## Practice Points

- Cryptococcosis is an important cause of morbidity and mortality in immuno-compromised patients and is the second most common fungal infection complicating AIDS.
- Various diagnostic methods such as conventional, serological and molecular tests are available.
- The sensitivity of PCR, antigen detection test and microscopy was found to be 100%, 89.19% and 78.4% respectively while the specificity of these tests was 82.43%, 85.14% & 90% respectively.
- The sensitivity of PCR test was significantly higher than tother tests. High prevalence of cryptococcal CNS infections in HIV-infected patients underscores the importance of utilizing this test in routine diagnosis of suspected cryptococcal infections.
- The present study indicates that PCR can be used as a rapid technique to detect *C. neoformans* in CSF and should be included in the routine testing protocols.

**Correspondence:** Sujata Dharmshale, Assistant Professor, Department of Microbiology, B. J. Govt. Medical College and Sassoon General Hospital, Pune, Maharashtra State, India. Email: sujatadharmshale@rediffmail.com.

South East Asia Journal of Public Health 2014;4(2):31-35.  DOI: http://dx.doi.org/10.3329/seajph.v4i2.23692

to evaluate the role of polymerase chain reaction in detection of cryptococcal meningitis in a clinical laboratory.

## Materials and Methods

A cross-sectional prospective study was carried out in Sassoon General Hospital, a large tertiary care centre in Pune, India, during April 2009 to February 2012. Ethical approval of the institute was obtained prior to the study. A total 111 cerebrospinal fluid (CSF) samples were collected from patients admitted with clinically suspected cryptococcal meningitis. All patients were screened for HIV as in the State Reference Laboratory of Microbiology Department at Pune, India as per national guidelines. The test kits used were: COMBAIDS® [(HIV1/2 immunoblot assay) Span Diagnostics Ltd India.], PAREEKSHAK® HIV1/2 Triline card [(immunochromatography assay) Bhat Bio-Tech India (P) Ltd.] and - AIDSCAN® HIV-1/2 TRISPOT test kit [(immune concentration based assay) Bhat Bio-Tech India (P) Ltd].[8]

CSF samples from 50 patients with definitive bacterial meningitis were included as controls. 1-3ml of the CSF sample was collected from all patients by lumbar puncture with all sterile precautions. The CSF was centrifuged at 1000 rpm for 15minutes. The supernatant of the CSF sample was transferred to a 1.5 ml small screw capped plastic vials for serological test for cryptococcal antigen detection. The deposit was used for microscopy, culture and Polymerase Chain Reaction (PCR).

Microscopical examination was performed by putting one drop of centrifuged CSF was taken and a drop of 10% Nigrosin on a clean glass slide and then wad covered with a glass cover slip. This was observed under the 40X high power light microscope to demonstrate the presence of capsulated yeasts. An air dried and heat fixed smear was stained with Gram's stain and was observed for Gram positive spherical encapsulated yeast cells with narrow buds.

Two slants of Sabouraud's Dextrose agar (SDA) were used of which one slant had no antibiotics and another had chloramphenicol (0.05mg/ml) and cycloheximide (0.5mg/ml). One blood agar was also inoculated. One set of media was incubated at 37°C and at 30°C. All media were observed daily for the growth till 21 days. If cream colored circular dome shaped mucoid colonies seen then these were further confirmed by gram stain, negative staining, growth at 37°C, urea hydrolysis test using Christensen's urea medium, and phenol oxidase production using Niger seed agar.[9]

The antigen detection was carried out by using CALAS (Meridian Bioscience, Inc., Cincinnati Ohio). The kit consists of latex particles coated with anti-cryptococcal polyclonal globulin. CSF samples were inactivated by keeping in boiling water beaker on tripod stand for five minutes to minimize any specific interference. The vials were allowed to cool and then the test performed as per manufacturer's instructions. The presence of visible agglutination when the latex particles were mixed with the CSF was taken to be a positive test.

DNA was extracted from the CSF by in house Silica-Guanidinium thiocyanate method.[10] The DNA obtained was amplified using specific primers CN4/CN5.[11] Positive control used was DNA extracted from *C. neoformans* ATCC strain 32045. *C. neoformans* specific primers as described by Mitchell *et al* were used.[12] These are: Forward: CN5 - 3'GAA GGG CAT TGT TTG AGA G 5' highly conserved sequence which overlaps with the 3'end of the 5.8S rDNA gene of *Filobasidiellla neoformans*. Reverse CN4 - 5'ATC ACC TTC CCA CTA ACA CATVT 3' [(Internal transcribed spacer (ITS) sequences of *F. neoformans* and *F. depauperata* (with substitution at three bases) BioResource Biotech Pvt. Ltd.]. These primers are 136bp long.

A final volume of 25µl, consisting of extracted DNA 02.0 µl, PCR master mix 12.5 µl (MME22L, MM-Bioscience Genei co), forward and reverse primers 02.5 µl and double distilled water 08.0 µl was prepared. Amplification of DNA was done by using thermo cycler (Applied Biosystem, USA).

A single cycle hot start at 95°C for 7 minutes was followed by 40 cycles at 95°C for 45 seconds and then annealing at 60°C for 1 min followed by extension at 72°C for 1.5min. The final extension temperature was set at 72°C for 7min.[11] The final PCR product was analyzed by Gel electrophoresis using 1% agarose, a sample loading dye (bromophenol blue and cyanol). A DNA marker/ladder of 200bp was also set up on the gel. After completion of the run gel was visualized (Figure 1).

The diagnostic statistics such as sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of tests were calculated considering culture as gold standard method. Mc Nemar's test was used to find out the significant difference in diagnostic tests in comparison with culture.

## Results

A total of 111 cerebrospinal fluid (CSF) samples were collected from patients admitted with clinically suspected cryptococcal meningitis of which 89 (80.18%) of the patients enrolled in the study were infected with HIV. No other immune-suppressive conditions such as corticosteroid therapy, cancer, diabetes mellitus and systemic lupus erythemaous were identified in other patients. The age range of the study patients was 4 to 65 years. The mean±SD for male and female was 37.7±9.35 and 32.6±9.14. The male and female ratio was 1.8:1. Three children were also included in the study group (Table 1).

The incidence of cryptococcal meningitis in the present study was 49.54% by using either smear/CALAS/ culture/PCR positive. *C. neoformans* was cultured from 37 patients i.e. 33.33% while *Trichosporon* spp and *Rhodotorulla* spp was cultured in two patients. A total

of 36 (32.43%) cases were positive by microscopy, 44 (39.63%) cases by the cryptococcal antigen test and 50 (45.05%) cases were positive by PCR (Table 2). The difference in detection rate of smear and antigen detection was not significant for microscopy ($p=1$) and for CALAS ($p>0.05$). The PCR was significantly better at detecting cryptococcal meningitis ($p<0.001$). All tests were negative in CSF collected from definite bacterial meningitis cases as controls.

The sensitivity of PCR, antigen detection test and microscopy was found to be 100%, 89.19% & 78.40% respectively while the specificity of these tests was 82.43%, 85.14% & 90% respectively. The positive predictive value of the PCR was 74% and the negative predictive value was 100% (Table 3).

## Discussion

The HIV pandemic has resulted in a surge of patients with cryptococcal meningitis both in developing and developed countries.[2] Early Flucanazole therapy gives a better outcome.[13] Thus it is imperative that the laboratory assists in the early diagnosis of this condition which will help in further patient management. In the present study the common age group affected was 21-40 years (72.06%) and a male predominance was observed. This was comparable with studies from North India and South India in which they reported >69%.[14,15] Culture is the gold standard method for diagnosis.[7,16] However, it takes 72 hrs to a month to grow and usually requires a large volume of sample.[7,16] Culture positivity in patients clinically diagnosed as cryptococcal meningitis has been reported to range from 2.7% to 100%.[14,17,18] In the present study it was found 33.03 %.

Direct microscopy on the CSF is a rapid and cost effective method performed by most laboratories.[19] In the present study, only 32.43% of CSF samples from suspected cryptococcal meningitis cases could be detected by this method. Microscopy positivity in cryptococcal meningitis has ranged from 50-90%.[7,15-17] The results of microscopy are dependent on the skill of the microscopist and are positive only if more than $10^3$ cells/ml of CSF are present.[11]

The latex agglutination test has been reported to be more sensitive than microscopy.[7,16] However, it has some drawbacks, false positive and false negative results in CSF as well as in serum have been reported.[20] In the present study, false positivity was obtained in 11 samples of which one grew as *Trichosporon spp*. Studies from Delhi by Saha *et al.*[17,7] have also reported 1.3% and 1.6% false positivity. Other reasons for false positivity are presence of rheumatoid factor, agar syneresis fluid, cross reactivity with *Capnocytophaga canimorsus* DF-2 bacillus), *Stomatococcus mucilaginosus* or *Klebsiella pneumonae*, non-specific reactivity in HIV infected patients, interferences starch, disinfectants and soap.[21]

Molecular tests have been developed for detection of nucleic acid directly in the biological samples[11] Some of the techniques being used for diagnosis are: PCR, in-situ hybridization technique using DNA probes, micro array technology.[22] The conventional PCR and

**Table 1:** Suspected cryptococcal meningitis cases in patients with different age categories

| Age categories | Total patients (n=111) | | Total |
|---|---|---|---|
| | Male | Female | |
| 1 to 12 years | 1 (0.9%) | 2 (1.8%) | 3 (2.70%) |
| 13 to 20 years | 2 (1.8%) | 3 (2.7%) | 5 (4.50%) |
| 21 to 30 years | 7 (6.3%) | 12 (10.82%) | 19 (17.12%) |
| 31 to 40 years | 43 (38.74%) | 19 (17.12%) | 62 (55.86%) |
| 41 to 50 years | 14 (12.61%) | 4 (3.6%) | 18 (16.21%) |
| 51 years and above | 4 (3.6%) | 00 | 4 (3.60%) |

**Table 2:** Test results of CSF in HIV reactive and non-reactive patients

| Test | Culture Positive | Culture Negative | Culture Contaminated | Total (n=111) |
|---|---|---|---|---|
| Microscopy Positive | 29 (26.12%) | 06 (5.41%) | 01 (0.9%) | 36 (32.43%) |
| Microscopy Negatives | 8 (7.2%) | 64+2*+1** (60.36%) | 00 | 75 (67.56%) |
| CALAS Positive | 33 (29.72%) | 10+1** (9.91%) | 01 (0.9%) | 45 (39.63%) |
| CALAS Negative | 4 (3.6%) | 60+2* (55.85%) | 00 | 66 (59.46%) |
| PCR Positive | 37 (33.33%) | 12 (10.81%) | 01 (0.90%) | 50 (45.05%) |
| PCR Negative | 00 | 58+2*+1** (54.95%) | 00 | 61 (54.95%) |

*Rhodotorulla spp; ****Trichosporon spp*

**Table 3:** Evaluation of diagnostic tests for Cryptococcal meningitis

| Test | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| Microscopy | 78.4% | 90.0% | 80.56% | 80.33% |
| CALAS | 89.19% | 85.14% | 75.0% | 94.03% |
| PCR | 100% | 82.43% | 74.0% | 100.0% |

PPV: Positive predictive value; NPV: Negative predictive value

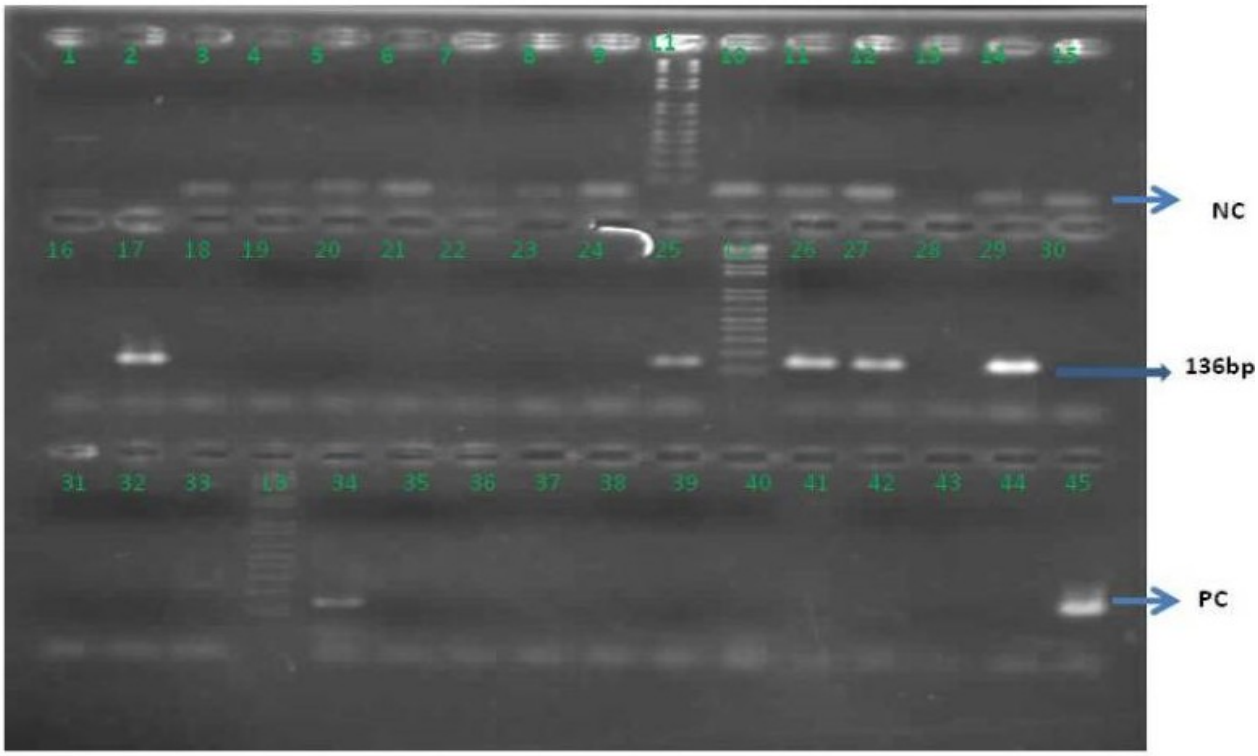


**Figure 1**: Gel Doc Image of PCR products
Lane no. 1-14, 16--44 are CSF sample, Lane 15-Negative Control, Lane 45-Positive Control. L1, L2 and L3 are 100bp DNA marker

the semi-nested PCR are diagnostic tools being increasingly used in fungal diagnosis for better management of patient.[11,22,23] PCR has been used for detection of *C. neoformans* by targeting 18S, 28S, or ITS (internal transcribed spacer) and 5.8S ribosomal DNA (rDNA).[12,23] In the present study the region ITS and 5.8S rRNA of *C. neoformans* was amplified as described by Mitchell *et al.*[12] which is species specific that means not amplifying other yeasts. This has been documented by Paschoal *et al*.[12]

In the present study, PCR positivity was 45.05% in suspected cryptococccal meningitis (Table 3). Compared to microscopy and antigen detection, PCR had a higher sensitivity. The sensitivity and specificity of PCR varies depending on which region of the *C. neoformans* is targeted. Other studies reported sensitivity 86-100% and specificity 100%.[7,11,20,23]

Using the same primers, Paschoal *et al.*[11] have detected PCR positive in 52/56 CSF samples from cryptococcal meningitis diagnosed clinically as well as microbiologically. However Saha *et al*.[7] have obtained 100% sensitivity and specificity of PCR by amplifying 278 bp region of 18S rDNA using nested primers for FungusI/II. Other studies have also reported that PCR is a more sensitive and efficient tool for diagnosis of neurocryptococcosis.[22-26] Its detection limit is reported to be 10 cells per ml.[7]

## Conclusion

The present study indicates that the Polymerase Chain Reaction (PCR) can be used as a rapid technique to detect *C. neoformans* in CSF. It should be incorporated into the routine diagnostic algorithm of patients with cryptococcal meningitis.

## Acknowledgement

The authors like to acknowledge Dr Shouche Y, Scientist 'G', Microbial Culture Collection (MCC), National Center for Cell Science (NCCS), NCCS Complex, Pune University Campus for allowing us to use their thermocycler for PCR.

## Conflict of interest

The author has declared that there is no competing interest.

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