Original Article

Assessment of A Real-time PCR Method to Detect Human Non-Cryptococcal Fungal Meningitis

Parisa Badiee PhD•[1], Abdolvahab Alborzi MD[1]

Abstract

Background: The signs and symptoms associated with fungal meningitis are similar to those seen with more common bacterial infections. In this study, we investigate whether *Aspergillus* or *Candida* DNA can be detected in cerebrospinal fluid (CSF) samples from patients suspected of fungal meningitis using real-time PCR assay.

Methods: From April 2007 to November 2009, we evaluated CSF samples and sera from patients with risk factors for cerebral fungal meningitis in Nemazi Hospital, Shiraz University of Medical Sciences, Iran, by real-time PCR assay and routine mycological studies (direct microscopy examination and culture). Two CSF and two serum samples from each patient were examined.

Results: CSF and serum samples from 38 patients (total: 152) suspected of fungal meningitis were examined. India ink staining and KOH smear were negative for all patients. *C. albicans* was isolated from two CSF samples. There were ten patients with positive real-time PCR results in their CSF samples: three patients had *C. albicans*, one with *C. glabrata*, four with *Aspergillus* species and two with both *C. albicans* and *Aspergillus* species DNA. Four patients had positive serum results for *Aspergillus* or *Candida* infections.

Conclusion: Considering the findings, it seems that molecular examination can help in the diagnosis of fungal meningitis in patients with clinical and radiological presentations. Further studies should be conducted in other regions and settings to confirm these findings.

Keywords: Fungal meningitis, *Aspergillus* spp., *Candida albicans,* real-time PCR, early diagnosis

Introduction

Fungal infections involving the central nervous system (CNS) are life-threatening conditions. *Candida* and *Aspergillus* species, as the most prevalent agents, can involve the CNS in special situations.[1–3]

Candidial infection of the cerebrospinal fluid (CSF) is an uncommon manifestation but may have a usual occurrence in immunocompromised patients in addition to those treated with broad spectrum antibiotics and steroids, and can be associated with intravenous hyperalimentation following surgical manipulation, mucosal surfaces, colonized with *Candida* species. *Candida* meningoencephalitis is seen in patients with hematological malignancy, neurosurgery, premature neonates and those with intravascular catheters.[3] The attributable mortality rate for meningo-encephalitis caused by *Candida* species is 10% – 30%.[4]

The prevalence of *Aspergillus* CNS localization at autopsy has been reported to be 23% – 59%[5] and *in vivo* it is 6% – 7%.[5] Meningitis caused by *Aspergillus* species is very rare and the attributable mortality rate for brain abscesses due to *Aspergillus* is 80% – 96%.[6,7] This infection presents in high risk patients such as those with acute myeloid leukemia, hematologic stem cell and solid organ transplant recipients, those under treatment with steroids and patients infected with human immunodeficiency virus.[3,5]

The syndrome often manifests itself with fever and headache that may be present for several weeks before a diagnosis is established. A rapid and accurate diagnosis is important for effective early treatment.

Authors' affiliations: [1]Prof. Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
•Corresponding author and reprints: Parisa Badiee PhD, Prof. Alborzi Clinical Microbiology Research Center, Nemazi Hospital, Zand Ave., Shiraz, Iran. Tel: +98-711-647-4304, Fax: 0098-711-6474303, E-mail: Badieep@yahoo.com.
Accepted for publication: 1 March 2011

Conventional diagnostic kits that can detect fungal antigens or antibodies as well as CSF culture are inadequate for early diagnosis because of the lack of high sensitivity or specificity. In recent years molecular methods have been developed in order to facilitate the diagnosis of invasive fungal infections. Several PCR assays have been developed to detect fungi in blood and bronchoalveolar lavage (BAL) fluid samples,[8,9] but the experience for the diagnosis of fungal infections in CSF samples by PCR remains limited.

In this study, we investigate whether *Aspergillus* or *Candida* DNA can be detected in CSF samples from patients with cerebral infections unresponsive to antibacterial therapy. We have used the real-time PCR assay, which has been clinically and experimentally validated for blood samples.

Materials and Methods

From April 2007 to November 2009, in a descriptive study, we used real-time PCR to evaluate CSF samples and sera from 38 patients with risk factors and suspected diagnosis of fungal meningitis in the Clinical Microbiology Research Center, Nemazi Hospital, Shiraz University of Medical Sciences, Shiraz, Iran. The risk factors for these patients were surgery, prolonged antibiotic therapy, and according to European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group[10] the prolonged use of corticosteroids (transplantation), meningeal enhancement on MRI or CT, and focal lesions on imaging indicative of CNS infection.

Blood and CSF cultures from these patients were negative for microbial agents and patients were unresponsive to a several day course of antibacterial agents. Upon receipt of patients' CSF samples, clinical samples (if available) that consisted of urine, bronchoalveolar lavage and sputum were also examined for fungal infections by culture on sabouraud-dextrose agar (Merck, Darmstadt,

Germany) and direct microscopic examination. Blood samples were cultured by bedside inoculation onto BACTEC medium (Becton-Dickinson, Sparks, MD, USA). Another CSF sample was obtained under sterile conditions by lumbar puncture and we collected two blood samples for molecular diagnosis. Thus, two CSF and two serum samples were examined from each patient. In cases with positive results for both samples, contamination was rejected and they were considered as documented cases. Samples were processed for routine mycological studies such as direct and India ink (for *Cryptococcus neoformans* capsules) microscopic smears and fungal culture on sabouraud-dextrose agar. Samples were then immediately processed for fungal PCR. As controls, CSF samples from 20 patients without risk factors for fungal infections were investigated by PCR.

For DNA extraction, QIAmp DNA Minikit (Qiagen, Hilden, Germany) was used in accordance with the manufacturer's recommendations. The taq-man probes were fluorescence labeled with 6-carboxy-fluorescein (FAM) reporter dye and 6-carboxy-teremethyl-rhodamine (TAMRA) as the quencher. Fungal DNA was detected by real-time PCR assay, as described previously. The fungal amplification primers for *Candida* were designed by Shin et al.[11] (forward primer: 5`-GCA TCG ATG AAG AAC GCA GC-3`; reverse primer: 5`-TCC TCC GCT TAT TGA TAT GC-3`). The fluorogenic probes for *C. albicans* (5`-AT TGC TTG CGG CGG TAA CGT CC-3`), *C. tropicalis* (5`-AA CGC TTA TTT TGC TAG TGG CC-3`), *C. parapsilosis* (5`-AC AAA CTC CAA AAC TTC TTC CA-3`), and *C. glabrata* (5`-TA GGT TTT ACC AAC TCG GTG TT-3`) were investigated. The fungal DNA primers and probe for amplification were used for *Aspergillus* according to Kami et al.[12] that were hybridized sequence for all *Aspergillus* species.

Sequences of PCR primers and probes were selected on the basis of the sequences of fungal 18S rRNA genes in the GenBank database. The forward primer was 5-TGGTGGAGTGATTT-GTCTGCT-3` and the reverse primer was 5`-CTAAGGGCATCA CAGACCTG-3`. The taq-man probe was: FAM 5'TCGGCCCT-TAAATAGCCCG GTCCGC-3'TAMRA.[12] Primers and taq-man probes were obtained from Metabion (Deutschland). Extracted DNA from patients and standard samples with PCR master mix (Roche, Branchburg, New Jersey, USA) and 0.2 µmol/L of each primers and probs of both *Candida* and *Aspergillus* species were analyzed using the Gene Amp 7500 sequence detection system (Applied Biosystems).

Thermal cycling conditions consisted of heating at 94°C for 10 minutes, which preceded a two-stage temperature profile of 30 seconds at 95°C and 90 seconds at 60°C for 40 cycles.[13] To minimize the risk of contamination, all samples were handled under sterile conditions in a laminar flow cabinet. DNA from *A. fumigatus* and *C. albicans* isolates were amplified with both primers and inserted into a pCR2.1 TA cloning vector (Invitrogen). We used these plasmids as the standard in this study. To determine the detection limit of the assay for fungal pathogens in the CSF, quantification of *A. fumigatus* and *C. albicans* DNA was performed with a serially diluted standard in the range of $10^1 - 10^8$ copies/well (Figure 1).

The Ethics Committee of the Clinical Microbiology Research Center at Shiraz University of Medical Sciences reviewed and approved this study. Patients provided written informed consents before participating in the study.

Results

Cerebrospinal fluid and serum samples from 38 patients suspected of fungal meningitis were examined by real-time PCR assay for both *Aspergillus* and *Candida* meningitis. Patients had histories of head trauma (car accident), bacterial brain and shunt infections (unresponsive to antibacterial agents), and surgery for tumor. Clinical signs such as fever, headache, nausea, nuchal rigidity, mental status changes, slurred speech and radiological signs such as inflammatory lesions with abscesses were present in patients. CSF analysis showed a mononuclear or neutrophilic pleocytosis, an elevated protein concentration and reduced glucose concentration. India ink staining and KOH smear were not positive in any of the patients. *C. albicans* were isolated from two CSF samples and two urine fungal cultures. None of the fungal blood cultures was positive.

The limitation of PCR assay was 10 copies/well. None of the control samples was positive. There were ten patients with positive results in their CSF samples: three patients had *C. albicans*,

Table 1. Clinical characteristics of patients with positive PCR results.

Patient No.	Age/ sex	Background	Risk factors	Blood suger /CSF suger and protein	WBC CSF	PCR serum	CSF PCR result	GCS	Outcome
1	46/M	Recurrent meningitis	Surgery ABT	105/25/96	100	*C.alb- Asp-*	*C.alb + Asp-*	15/15	Lived
2	5/M	Astrocytoma	Surgery ABT	90/38/297	260	*C.alb- Asp-*	*C.alb+ Asp+*	15/15	Lived
3	25/M	Motor accident	Surgery ABT	131/23/138	105	*C.alb+ Asp-*	*C.alb+ Asp-*	4/15	Died
4	38/F	Kidney transplant	Im.drug	111/38/105	90	*C.alb+ Asp+*	*C.alb+ Asp+*	6/15	Died
5	7/F	Astrocytoma	Surgery ABT	234/74/145	120	*Can- Asp-*	*Can- Asp+*	15/15	Lived
6	17/M	Motor accident	Surgery ABT	68/19/143	118	*Can- Asp-*	*Can- Asp+*	3/15	Died
7	39/M	Bacterial meningitis	Drug abuser ABT	113/5/130	750	*Can- Asp+*	*Can- Asp+*	3/15	Died
8	7/M	Hydrocephaly	Sh. infect ABT	86/14/284	80	*C. glab- Asp-*	*C. glab+ Asp-*	15/15	Died
9	26/F	HIV	HIV	144/20/200	3000	*C.alb+ Asp-*	*C.alb+ Asp-*	6/15	Lived
10	49/M	Ventriculostomy	Sh. infect ABT	90/30/110	4	*Can- Asp-*	*Can- Asp+*	3/15	Died

M=male; F=Female; Im.drug=immunosuppressive drugs; Sh. infect=shunt infection; Asp= Aspergillus; Can= Candida; C. glab=*C. glabrata*; ABT=antibiotic therapy; GCS=Glascow Coma Score.

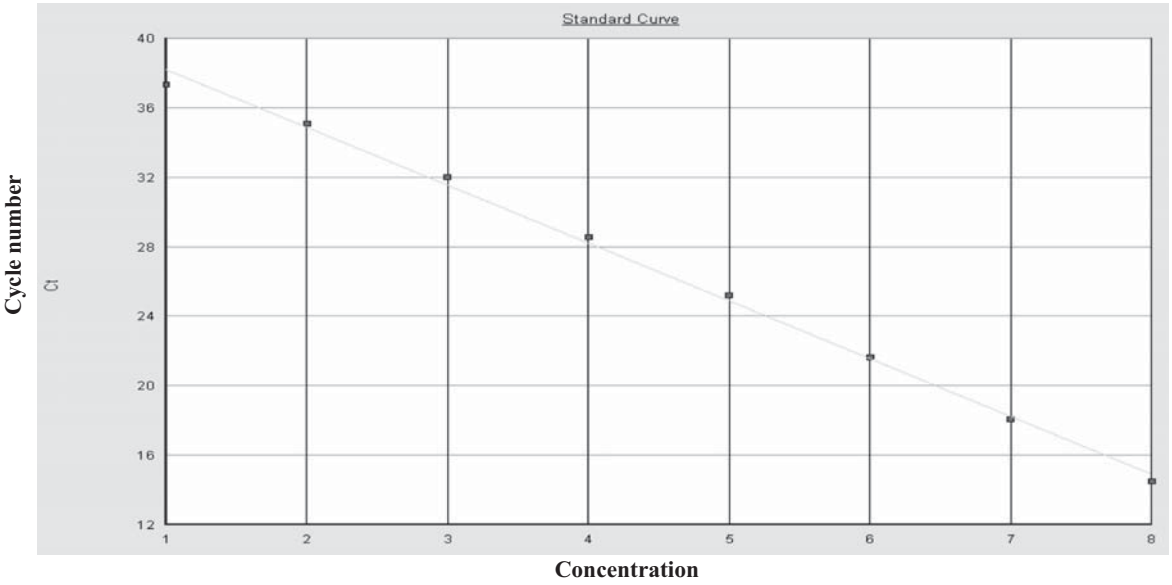

Figure 1. Standard curve for *Aspergillus* real-time PCR (10^1-10^8 copies/well).

one with *C. glabrata*, four with *Aspergillus* species, and two with both *C. albicans* and *Aspergillus* species DNA. Four patients had positive sera results for *Aspergillus* or *Candida* infections. Clinical characteristics of the patients and PCR results are shown in Table 1. Once the fungal infection was diagnosed, antifungal therapy was initiated. Unfortunately six patients died, most probably due to late diagnosis (Table 1).

Discussion

Unfortunately all brain fungal infections have the same signs and symptoms: fever (40% – 80%), cerebral hemorrhage (35% – 50%), altered mental status (30% – 50%), hemiplegia/paresis (19% – 35%), cranial nerve abnormalities (30%), epilepsy/seizure (5% – 25%), nausea/vomiting (10% – 20%), ptosis (10%), and headache (10%).[3,14] Signs and symptoms associated with fungal meningitis are similar to those seen with the more common bacterial infections. Prolonged and severe neutropenia, high dose corticosteroid therapy and treatment for solid organ transplantation predispose patients to fungal meningitis. The infection can also follow head trauma or surgery.[15]

Histopathology is one of the most reliable laboratory methods to prove the disease, but biopsies are invasive diagnostic tools. When biopsy is not possible, CSF analysis can prove useful for diagnosis although there is no differential in CSF analysis between bacterial and fungal meningitis. A glucose level below 40 mg/mL, high levels of protein and lactate de hydrogenase, and increasing in polymorphonuclear leukocytes (neutrophils) are significant conditions in both infections.[16,17]

For diagnosis, routine methods such as CSF culture are not sensitive enough. Fungal cultures are positive in more than 95% of *Cryptococcus neoformans* cases and in 66% of candidal meningitis cases. Other fungi (particularly *Aspergillus* and *Zygomycetes*) are less likely to be culture positive.[18] Khan and co-workers have reported a case of cerebral aspergillosis that was diagnosed by the detection of *A. flavus*-specific DNA in the brain biopsy and serum specimens. Despite the presence of dichotomously branched septate hyphae in the brain biopsy, the culture remained negative.[19] Barenfanger and co-workers[20] examined data from the 1,225 samples of CSF which were cultured for both bacterial and fungal infections. The researchers suggest that, in areas where *Candida* and *Cryptococcus* cause the vast majority of fungal meningitis, the combined use of a cryptococcal antigen test and bacterial cultures of CSF could replace routine CSF fungal cultures. In this study, fungal cultures were positive for *C. albicans* in only two CSF samples and none of the samples were positive for *Aspergillus*. Serological tests (detection of antigen or antibody) are not reliable. Komatsu and associates have described a patient with osteosarcoma in whom a brain abscess developed after autologous peripheral stem cell transplantation. Serologic markers of fungal infection were negative, but fungal DNA was detected in the CSF by panfungal polymerase chain reaction assay using primers derived from fungal 18S ribosomal RNA genes.[21] To circumvent these problems, a PCR system of detecting fungal DNA has been developed recently. Since *Candida* and *Aspergillus* species are responsible for most of the invasive fungal infections in immunocompromised patients, several protocols with specific primers have been established for the detection of DNA of these fungi.[12,22]

Indeed, the majority of cases of CNS candidiasis are associated with disseminated or invasive candidiasis. Another form of candidal CNS infection occurs as a postoperative complication of neurosurgical procedures, especially ventriculo-peritoneal shunt placement. *Candida* meningitis is the most frequent clinical manifestation of invasive CNS candidiasis.[4] According to the results (Table 1) in the present study, PCR was positive in 6 out of 10 patients for *Candida* who had risk factors.

Candida CNS infections are usually due to *C. albicans*, but other *Candida* species might be involved in CSF complications, with *C. glabrata* as a rare cause of CNS infection.[23,24] In the present study all the *Candida* infections were caused by *C. albicans,* with the exception of one that was caused by *C. glabrata.*

The pathways of spread to the CNS may be direct (skull fracture, neurosurgical device), olfactory (via sino-nasal) and hematogenous (spread from lungs, blood dissemination). In 90% – 95% of cases, brain abscess is secondary to a pulmonary localization but in the remaining 5% – 10% of cases, CNS abscess was a unique manifestation.[24] In the current study, four patients with disseminated fungal infections had positive PCR results for both serum and CSF, but in

localized infections (six cases) only the CSF results were positive.

The findings in this descriptive study indicate that molecular assay is useful for the diagnosis of CNS fungal infection and identification of the pathogens in CSF samples. To confirm these findings, further studies seem necessary.

Funding source

This work was supported by Professor Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

Conflict of interest statement

There were no conflicts of interest.

Acknowledgements

We would like to thank H. Khajehei PhD for linguistic copy editing.

References

1. Ruhnke M, Kofla G, Otto K, Schwartz S. CNS aspergillosis: recognition, diagnosis, and management. *CNS Drugs.* 2007; **21:** 659 – 676.
2. Marinovic T, Skrlin J, Vilendecic M, Rotim K, Grahovac G. Multiple *Aspergillus* brain abscesses in immuno-competent patient with severe craniofacial trauma. *Acta Neurochir (Wien*). 2007; **149:** 629 – 632.
3. Pagano L, Caira M, Falcucci P, Fianchi L. Fungal CNS infections in patients with hematologic malignancy. *Expert Rev Anti Infect Ther.* 2005; **3:** 775 – 785.
4. Pappas PG, Rex JH, Sobel JD, Filler SG, Dismukes WE, Walsh TJ, et al. Infectious Diseases Society of America. Guidelines for treatment of candidiasis. *Clin Infect Dis.* 2004; **38:** 161 – 189.
5. Kleinschmidt-DeMasters BK. Central nervous system aspergillosis: a 20-year retrospective series. *Hum Pathol*. 2002; **33:** 116 – 124.
6. Lin SJ, Schranz J, Teutsch SM. Aspergillosis case-fatality rate: systematic review of the literature. *Clin Infect Dis.* 2001; **32:** 358 – 366.
7. Pagano L, Girmenia C, Mele L, Ricci P, Tosti ME, Nosari A, et al. Infections caused by filamentous fungi in patients with hematologic malignancies. A report of 391 cases by GIMEMA Infection Program. *Haematologica.* 2001; **86:** 862 – 870.
8. Spiess B, Buchheidt D, Baust C, Skladny H, Seifarth W, Zeilfelder U, et al. Development of a LightCycler PCR assay for detection and quantification of *Aspergillus fumigatus* DNA in clinical samples from neutropenic patients. *J Clin Microbiol*. 2003; **41:** 1811 – 1818.
9. Badiee P, Alborzi A, Shakiba E, Ziyaeyan M, Pourabbas B. Molecular diagnosis of *Aspergillus* endocarditis after cardiac surgery. *J Med Microbiol*. 2009; **58:** 192 – 195.
10. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis.* 2008; **46:** 1813 – 1821.
11. Shin JH, Nolte FS, Holloway BP, Morrison CJ. Rapid identification of up to three *Candida* species in a single reaction tube by a 5' exonuclease assay using fluorescent DNA probes. *J Clin Microbiol.* 1999; **37:** 165 – 170.
12. Kami M, Fukui T, Ogawa S, Kazuyama Y, Machida U, Tanaka Y, et al. Use of real-time PCR on blood samples for diagnosis of invasive aspergillosis. *Clin Infect Dis*. 2001; **33:** 1504 – 1512.
13. Maaroufi Y, De Bruyne JM, Duchateau V, Georgala A, Crokaert F. Early detection and identification of commonly encountered Candida species from simulated blood cultures by using a real-time PCR-based assay. *J Mol Diagn*. 2004; **6:** 108 – 114.
14. Mattiuzzi G, Giles FJ. Management of intracranial fungal infections in patients with haematological malignancies. *Br J Haematol*. 2005; **131:** 287 – 300.
15. Gunaratne PS, Wijeyaratne CN, Chandrasiri P, Sivakumaran S, Sellahewa K, Perera P, et al. An outbreak of Aspergillus meningitis following spinal anaesthesia for caesarean section in Sri Lanka: a post-tsunami effect? *Ceylon Med J*. 2006; **1:** 137 – 142.
16. LeFever KJ. *Handbook of Laboratory and Diagnostic Tests*. 4th ed. Upper Saddle River, NJ: Prentice Hall; 2001.
17. Smith GP, Kieldsberg CR. *Cerebrospinal, Synovial, and Serous Body Fluids*. Philadelphia, PA: W. B. Saunders; 2001.
18. Treseler CB, Sugar AM. Fungal meningitis. *Infect Dis Clin North Am.* 1990; **4:** 789 – 808.
19. Khan ZU, Ahmad S, Mokaddas E, Said T, Nair MP, Halim MA, et al. Cerebral aspergillosis diagnosed by detection of *Aspergillus flavus*-specific DNA, galactomannan and (1-->3)-beta-D-glucan in clinical specimens. *J Med Microbiol.* 2007; **56:** 129 – 132.
20. Barenfanger J, Lawhorn J, Drake C. Nonvalue of culturing cerebrospinal fluid for fungi. *J Clin Microbiol.* 2004; **42:** 236 – 238.
21. Komatsu H, Fujisawa T, Inui A, Horiuchi K, Hashizume H, Sogo T, et al. Molecular diagnosis of cerebral aspergillosis by sequence analysis with panfungal polymerase chain reaction. *J Pediatr Hematol Oncol.* 2004; **26:** 40 – 44.
22. Badiee P, Kordbacheh P, Alborzi A, Zakernia M, Haddadi P. Early detection of systemic candidiasis in the whole blood of patients with hematologic malignancies. *Jpn J Infect Dis.* 2009; **62:** 1 – 5.
23. Sánchez-Portocarrero J, Pérez-Cecilia E, Corral O, Romero-Vivas J, Picazo JJ. The central nervous system and infection by Candida species. *Diagn Microbiol Infect Dis*. 2000; **37:** 169 – 179.
24. Fernandez M, Moylett EH, Noyola DE, Baker CJ. Candidal meningitis in neonates: a 10-year review. *Clin Infect Dis.* 2000; **31:** 458 – 463.