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Utility of Real-Time PCR for detection of *Exserohilum rostratum* in body and tissue fluids during the multistate outbreak of fungal meningitis and other infections

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ABSTRACT

Exserohilum rostratum was the major cause of the multistate outbreak of fungal meningitis linked to contaminated injections of methylprednisolone acetate (MPA) produced by the New England Compounding Center (NECC). Previously, we developed a fungal DNA extraction procedure and broad range and *E. rostratum*- specific PCR assays, and confirmed the presence of fungal DNA in 28% of case-patients. Here we report the development and validation of a TaqMan® real-time PCR assay for detection of *E. rostratum* in body fluids, which we used to confirm infections in 57 additional case-patients, bringing the total number of case-patients with PCR-positive results for *E. rostratum* to 171 (37% of 461 case-patients with available specimens). Compared to fungal culture and the previous PCR assays, this real-time PCR assay was more sensitive: of 139 case-patients with identical specimens tested by all three methods, 19 (14%) specimens were positive by culture, 41 (29%) positive by the conventional PCR, and 65 (47%) positive by the real-time PCR. We also compared utility of the real-time PCR with the previously described beta-D-glucan (BDG) detection assay for monitoring response to treatment in case-patients with serially collected CSF. In most case-patients, only the incident CSF specimens were positive by real-time PCR while most subsequently collected specimens were negative, confirming our previous observations that BDG assay was more appropriate than real-time PCR for monitoring response to treatment. Our results also demonstrate that real-time PCR is extremely susceptible to contamination and its results should only be used in conjunction with clinical and epidemiological data.

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INTRODUCTION

Since September 2012, US state and local health departments, the Centers for Disease Control and Prevention (CDC), and the Food and Drug Administration have been investigating the largest documented health care-associated outbreak in the United States of fungal meningitis and other infections developed after epidural, paraspinal, or joint injections with contaminated methylprednisolone acetate (MPA) from a single compounding pharmacy(1-3). The major cause of this outbreak was *E. rostratum*, a plant pathogen that rarely causes disease in humans(4). Using culture, PCR and histopathology, this fungus was identified from cerebrospinal fluid (CSF), synovial fluid, abscess aspirates, fresh tissue, and formalin fixed paraffin embedded tissue samples from 153 case patients, as well as from unopened vials of two implicated lots of MPA (3). Previously, we developed a method for extracting free circulating fungal DNA from different types of body fluids and tissues from patients in this outbreak and used two PCR tests followed by DNA sequencing to detect fungal DNA in patient specimens(5). Using this approach, we were able to detect fungal DNA in 28% of case-patients (3). Although the sensitivity of PCR was considerably better than culture, which was only able to confirm 10% of cases, a large percentage of case-patients remained without laboratory confirmation of infection. We also investigated the utility of the Fungitell® assay for detection of beta-D-glucan (BDG) in CSF for diagnosis and monitoring response to treatment (6). Using this assay and a stratified sample of CSF specimens, we demonstrated that approximately 45% of CSF specimens from case-patients with negative PCR results had elevated levels of BDG. Our results as well as others also demonstrated that consistently elevated levels of BDG in CSF may indicate

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a relapse or persistent infection (6, 7). However, because the BDG assay is not species-specific, can cross-react with certain bacteria and drugs, and is prone to contamination, this test cannot be used to provide a definitive confirmation of fungal infection (6, 8). A real-time molecular beacon PCR assay for detection of *Exserohilum* has also been reported (9) and used to test blood from patients with a possible association with the outbreak (10).

Here, we report development of a quantitative real-time PCR assay using TaqMan® technology for detection of the ITS2 ribosomal DNA region of *E. rostratum*. We report sensitivity and specificity results of this novel real-time PCR assay and compare the utility of this assay with previously reported broad-spectrum PCR and BDG detection assays.

MATERIALS AND METHODS

Human subjects. This investigation was considered as an emergent public health response and therefore was not subject to review by the CDC's Institutional Review Board.

Case definitions. A proven case was defined as a probable case with evidence (by culture, histopathology, or molecular assay) of a fungal pathogen associated with the clinical syndrome. A probable case was defined as development of any of the following: meningitis of unknown etiology following epidural or paraspinal injection after 21 May 2012; posterior circulation stroke without a cardioembolic source and without documentation of a normal CSF profile following epidural or paraspinal injection after 21

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May 2012; osteomyelitis, abscess, or other infection (e.g., soft tissue infection) of unknown etiology in the spinal or paraspinal structures at or near the site of injection following epidural or paraspinal injection after 21 May 2012; or osteomyelitis or worsening inflammatory arthritis of a peripheral joint (e.g., knee, shoulder, or ankle) of unknown etiology diagnosed following joint injection after 21 May 2012 in a person who received an injection with preservative-free MPA that definitely or likely came from one of the following three lots produced by the New England Compounding Center (NECC): 05212012@68, 06292012@26, 08102012@51. These case definitions have been previously described (3, 11).

Clinical specimens. This report includes specimens received between October 2, 2012 and December 31, 2013. A total of 898 specimens from 471 case patients were tested including CSF, synovial fluid, abscess aspirates and tissue fluids. All of these specimens were previously tested with two conventional PCR assays as previously described (5). Fresh tissues, formalin-fixed paraffin-embedded tissue and isolates were excluded. Serially collected CSF from 20 case-patients whose specimens were previously tested for BDG detection were included to evaluate the utility of real-time PCR for monitoring response to treatment (6, 12).

DNA Extraction from CSF, synovial fluid and abscess aspirates. Body fluids such as CSF, synovial fluid, and abscess aspirates were processed as previously described (5). Briefly, cells and particulate matter were pelleted by centrifugation and removed, and free DNA was captured using the QIAamp UltraSens virus kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with modifications as described(5). The same DNA extractions were used for conventional and real-time

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PCR. DNA was stored at -80°C and thawed twice prior to testing. Early in the outbreak, some remnant samples that had not been kept frozen were sent for analysis. Otherwise, specimens were frozen as soon as possible after collection, were shipped on dry ice to CDC, and were kept at -80°C until testing.

DNA extraction from fresh-frozen tissue fluids. Fresh-frozen tissues were received from a variety of sources, including brain, meninges, epidural tissue, epidural abscess debridement, cyst, and bone in sterile saline. Specimens were centrifuged and the supernatants were processed using the body fluid protocol as described(5).

Real-Time PCR primers and probe. To select specific real-time PCR primers and probe, we used Mega 5.0 (13) to generate alignment between sequences from the ITS2 region of the *E. rostratum* outbreak strains and other fungi associated with the outbreak (14) as well as *E. rostratum* and 11 species of phylogenetically related and clinically relevant fungi, such as *Bipolaris* sp. and *Alternaria* sp, from GenBank. Regions specific to *Exserohilum* were selected and used to select probes and primers using Primer 3 (http://frodo.wi.mit.edu/) and *Exserohilum* ITS DNA strain (NCBI accession number JF819166.1). The real-time PCR primers were: EXS 1F, 5′-TTG TCT CTC CCC TTG TTG G -3′; EXS 1R, 5′-CCG CCC CGT GGA TTG GAA -3′ and the probe was: 5′- FAM-TTG GCA GCC GAC CTA CTG GTT TT -3′-BHQ1, where FAM is 6-carboxyfluorescein and BHQ is the Black Hole Quencher®. Specificity of the selected primer and probe sequences was tested by BLAST using the NCBI Nucleotide collection (nr/nt) database, and no significant similarity with any other reliably identified species of fungi was detected.

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Real-Time PCR Assay. A 25-µl reaction mixture contained 12.5 µl of 2X TaqMan® universal master mix (Applied Bio-Systems, Gaithersburg, MD), 0.2 µM of each primer, 0.1 µM of TaqMan® probe and 5 µl of the template DNA. The reactions were performed using a Rotor-Gene real-time instrument (QIAGEN RG 6000) with the following cycling conditions: an initial denaturation step at 95°C for 5 min, followed by 45 cycles of 95°C for 15 s , 54°C for 30 s and 72°C for 10 s with data collection in the FAM channel. Each run included one *Exserohilum* genomic template control and at least two no-template controls. All specimen DNAs were tested in duplicate.

Analytical specificity of the real-time PCR primers and probe. The analytical sensitivity of primers and probe was tested using *E. rostratum* DNAs extracted from 21 *E. rostratum* isolates from case-patients, 6 isolates from MPA vials and 7 *E. rostratum* strains from the CDC collection. We also tested DNA extracted from 12 non-*Exserohilum* fungal pathogens recovered from case-patients from this outbreak, such as *Alternaria alternata, Aspergillus fumigatus, Aspergillus terreus, Aspergillus tubingensis, Chaetomium sp., Cladosporium cladosporioides, Cladosporium sp., Epicoccum nigrum, Paecilomyces niveus, Penicillium panem, Scopulariopsis brevicaulis, Stachybotrys chartarum* (14), as well as other clinically relevant fungi, such as *Fusarium sp.,* and *Candida albicans*. In addition, we tested DNAs extracted from body fluids positive for other fungi by the conventional PCR, such as *Cladosporium cladosporioides, Cladosporium sp. Epicoccum nigrum, Alternaria sp.* and *Malassezia restricta*.

Determining limit of detection (LOD). PCR products were cloned into pCR™ 2.1 TA vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, linearized by digesting with *HindIII* (New England Bio Labs, Ipswich, MA) according to

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the manufacturer's instructions, purified using QIA Quick PCR purification kit (QIAGEN, Hilden, Germany) and used for preparing 10-fold serial dilutions containing 10^7–10 plasmid copies per reaction. A standard curve was generated using Rotor-Gene Q Series Software 2.0.2 (SA Biosciences QIAGEN) and used to calculate PCR efficiency and linear dynamic range; the experiment was performed in triplicate. The lowest concentration of DNA that could be reliably detected was considered the LOD of this method.

Estimating reproducibility. Assay reproducibility was tested by comparing Ct values obtained on three different days using replicate 10-fold serial dilutions of the linearized plasmid DNA and measured by calculating a coefficient of variation (CV) of the mean Ct values between runs. Precision was evaluated for each dilution point by comparing variability among triplicates and measuring CV of the mean Ct values within runs.

Determining diagnostic sensitivity and specificity. To calculate diagnostic sensitivity, we compared the performance of the real-time PCR assay with culture and previously developed broad-spectrum and *E. rostratum*–specific conventional PCR assays using previously described 139 CSF samples from proven and probable cases with known PCR and culture results (5). Positive control specimens were specimens that had demonstrated positive conventional PCR (N=41) and/or culture (N=19) results for *E. rostratum*. Diagnostic specificity was calculated using 66 negative control specimens from patients who were not considered cases in the outbreak. To test for the possibility of cross-contamination during sample handling, we tested 61 sterile distilled water samples that were processed along with the patient specimens and subjected to the same procedures for DNA extraction.

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RESULTS

Analytical specificity of the real-time PCR primers and probes. Identical PCR amplification patterns were obtained using DNA extracted from 34 different strains of *E. rostratum* (data not shown). No amplifications (Ct value 0) were obtained when DNA from 15 other fungal species or control human DNA was used.

Limit of detection and amplification efficiency. The limit of detection was approximately 10 copies per reaction, which corresponded to Ct value of 40 ± 0.3. Strong linear correlations (r^2>0.99) were obtained between Ct values and target copy numbers over a 7-log range (10-10^7 copies per reaction) with 98% amplification efficiency (Supplemental figure S1).

Reproducibility. Assay reproducibility was tested by using replicate 10-fold serial dilutions of the linearized plasmid DNA and intra- and inter assay variability was evaluated for each dilution point in triplicate on three different days. The coefficient of variation (CV) of the mean Ct values within and between runs was 0.20%-1.5% and 0.2%-2.9%, respectively (Table 1).

Diagnostic sensitivity of broad-spectrum PCR, real-time PCR and culture. Specimens from 139 case-patients were tested by culture, conventional PCR and real-time PCR to assess the diagnostic sensitivity of each method (Table 2). Sixty five of these specimens were positive by real-time PCR (47%), 41 were positive by the conventional PCR assays (29%) and 19 were positive by culture (14%) (5). All specimens positive by conventional PCR and/or culture were also positive by real-time PCR. Furthermore, 5 specimens that were positive by culture but negative by conventional PCR in previous testing also were positive by real-time PCR. In addition,

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19 specimens that were negative by both conventional PCR and culture were positive with real-time PCR (Table 2).

Diagnostic specificity of the real-time PCR. To determine the specificity of the assay, we tested 61 control water samples that were subjected to the same DNA extraction procedure as the patient specimens (5): for 54 (89% of 61) of these samples, no amplification was observed after 45 cycles (Ct value 0); however, a low level of *E. rostratum* DNA (Ct value 42-43) was detected in 7(11% of 61) samples (data not shown). Furthermore, we tested 66 CSF samples from patients not considered cases in the outbreak. For 56 (85% of 66) of these samples, no amplification was observed after 45 cycles of PCR (Ct value 0); however, low levels of *E. rostratum* DNA were detected (Ct value 41-45) in 10 (15% of 66) of these samples (data not shown). Based on these observations, Ct values ≤40 were considered positive, Ct values of 41- 45 were considered indeterminate, and Ct values of 0 (>45) were considered a negative result.

Results of real-time PCR Assay. Between October 2012 and December 2013, 898 CSFs, synovial fluids, abscess aspirates and tissue fluids from 471 case patients were tested by the broad-range and *E. rostratum*-specific conventional PCR assays and DNA sequencing (5). Of those, 888 specimens from 461 case-patients were available for real-time PCR testing. Ten samples tested previously by the conventional PCR assay were not tested by real-time PCR because of an insufficient quantity of specimen/DNA. A total of 209 samples (24% of all tested samples) collected from 171 case-patients (37% of 461 case-patients) were found positive for *E. rostratum* DNA using real-time PCR (Table 3). Positive results were obtained for 174 CSF from 138 case-patients (38% of 359 case-patients with CSF samples), 7 synovial/abscess fluids from 6 case-patients (21% of 30 case-patients with synovial specimens) and 28 tissue fluids from 27

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case-patients (33% of 82 case-patients with tissue fluids; Table 3). The median Ct value for the positive samples was 37.6. The vast majority of the real-time PCR positive specimens (193 of 209 (92%) positive specimens) were received in October-December of 2012.

Testing serial CSF samples. Results obtained by conventional PCR, real-time PCR and BDG levels in CSF were compared among 20 case patients with serially collected samples described previously (6) (Table 4). Both conventional and real-time PCR were more likely to detect fungal DNA in the incident CSF samples compared to later serially collected samples (Table 4). Specifically, incident CSF samples from 11 case-patients were positive by conventional PCR and 18 by real-time PCR. In addition, in Patients 5 and 17, real-time PCR detected *E. rostratum* DNA in two subsequent CSF specimens collected approximately one month after the first samples were collected. Furthermore, both conventional and real-time PCR detected *E. rostratum* DNA in the CSF of Patient 3 after he relapsed with meningitis (Table 4)(15). However, all three serially collected CSF samples from Patient 4, who developed soft tissue phlegmon, were positive by real-time PCR, while only the incident CSF from this patient was positive by the conventional PCR assay. All CSF with positive real-time PCR results also had elevated BDG levels (Table 4). Furthermore, in most case-patients, elevated BDG levels persisted for an additional 3-5 months when real-time PCR results were indeterminate or negative (Table 4).

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DISCUSSION

The 2012 multistate outbreak of fungal meningitis and other infections has been the largest iatrogenic outbreak in US history, and is still affecting the lives of hundreds of people. By October 23, 2013, in 20 states 752 cases were identified, resulting in 64 deaths. CDC laboratories were able to confirm fungal infections in 173 case-patients (33% of case-patients from whom specimens were submitted to CDC) using histopathology, PCR or culture (3, 14). In addition, with input from the Infectious Diseases Society of America and an advisory group of experts in clinical mycology, CDC developed treatment guidelines for physicians treating patients from the outbreak (11, 16). Although the recommended clinical management for both confirmed and probable cases in the outbreak was the same, there was a need to develop a more sensitive diagnostic test to better understand the etiology of this outbreak. Our previously developed conventional PCR assays were able to detect fungal DNA in 114 case-patients with available body fluid specimens (25% of case-patients for whom specimens were submitted to CDC)(5). Here, we report development of a novel real-time PCR assay for detection of *Exserohilum* DNA in case-patients from the outbreak and provide laboratory confirmation of *Exserohilum* infection in 57 additional case-patients.

Increased sensitivity of the real-time PCR assay compared to the conventional PCR was not surprising since real-time PCR did not rely on the visualization of PCR products on an agarose gel, and therefore, lower concentrations of PCR products could be detected with this method. We observed an excellent correlation between conventional and real-time PCR results, as all samples positive by conventional PCR

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were also found positive by real-time PCR. In addition, all samples positive by culture were also positive by real-time PCR, and 5 samples that were positive by culture but negative by conventional PCR were found positive by real-time PCR.

There are at least three limitations of this real-time PCR assay. First, this assay is specific to *Exserohilum* and cannot be used to detect other fungal DNA. Several other fungi have been implicated in the outbreak; however, their clinical significance remains to be determined (10, 14). Our results are consistent with previous observations that *E. rostratum* is the main etiological agent of this outbreak (10, 14), as its DNA was detected in body fluids or tissues of 37% of all tested case-patients. Second, extracting fungal DNA from patient specimens remains the main challenge for this molecular detection method, since the sensitivity of PCR depends on the efficiency of DNA extraction. Here we used a DNA extraction method that relies on purification of free-circulating fungal DNA from fluids, which provided the best recovery of fungal DNA from these outbreak samples (5); however, the effectiveness of this method needs to be further evaluated. Third, our results demonstrate that this real-time PCR assay is highly sensitive to contamination. Even miniscule amounts of contaminating DNA introduced during sample collection or handling can interfere with the PCR signal from the fungus causing infection; therefore, molecular results need to be interpreted in combination with clinical and epidemiological data and very low concentrations of DNA cannot be reliably linked to disease.

To minimize the effects of contamination and cross-contamination that occur during sample collection and handling, we used the following procedures. First, prior to use in the real-time PCR assay, we tested all reagents for the presence of fungal DNA.

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Surprisingly, we detected *Fusarium sp.* DNA in several lots of proteinase K, which is used for digestion of human cells and proteins. Second, when processing patient samples for DNA extraction, we included a water control sample with each batch of patient samples and subjected it to the same procedures used for processing patient specimens. When these control water samples were tested by real-time PCR, a low level of amplification (Ct value 42-43) was detected in 7(11%) of 61 samples indicating cross-contamination during the extraction.

Recently, there has been an increased interest in using PCR-based methods for diagnosis of fungal infections from this outbreak and in other patient populations (9, 10, 17, 18). Our data suggest that PCR results need to be carefully scrutinized and should only be considered in combination with clinical and epidemiological data. False positive PCR results caused by contamination during sample collection and processing have been reported by others (19-21) and our results support these observations. Reagent impurity, contamination during sample collection from skin flora and/or environment, as well as cross-contamination during DNA extraction and processing cannot be completely avoided, and therefore, amplification of fungal DNA from a biological sample does not always imply infection. For example, in this outbreak several other environmental fungi have been detected by PCR in CSF and other body fluids and tissues (14). Although some of these agents, such as *Cladosporium cladosporioides,* have been implicated in this outbreak, others, such as common wheat (*Triticum sp.*) or skin flora *Malassezia restricta*, which we PCR-amplified from CSF of several case-patients (data not shown), were unquestionably contaminants. Recently, Zhao et al used real-time PCR to detect the presence of fungal DNA in blood of patients exposed

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to contaminated MPA; however, the clinical significance of these findings remains unclear (10). Most of the fungi detected by Zhao et al, such as *Cryptococcus neoformans* or *Hortaea werneckii*, have never been implicated in this outbreak (3, 12, 14). Although *Aspergillus fumigatus* was isolated from this outbreak index case (22), extensive screening of more than 1000 specimens from more than 500 case-patients from this outbreak using broad-spectrum fungal PCR in our laboratory (14) as well as independent screening with *Aspergillus*-specific PCR at a Virginia hospital (12) failed to detect any evidence of *A. fumigatus* DNA in any patients other than the index case (3). Overall, our experience with PCR and real-time PCR suggests that, in the absence of other evidence, detection of trace amounts of fungal or any other foreign DNA in biological samples could be attributed to contamination and cannot be used as a sole argument for infection.

Previously, we tested the utility of BDG detection for monitoring response to treatment in serially collected CSF. Here we investigate the utility of real-time PCR for monitoring patient response to treatment. Our results demonstrate that although real-time PCR was able to detect *Exserohilum* DNA in a larger number of case-patients with serially collected CSF compared to conventional PCR, in most patients, only CSF collected during the early stages of disease were positive using this test. Specifically, *Exserohilum* DNA was detected by real-time PCR in 17(85%) of 20 case-patients with serially collected samples. Of those, in 13 (76%), only the incident specimens were positive, in 2 (12%, Patient 5 and 17) both incident as well as the subsequent samples collected one month after the initial diagnosis were positive, and in one patient (6%, Patient 3) who relapsed with meningitis, both the incident and the relapse CSF were

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positive by real-time PCR (Table 4). The only exception was Patient 4, who developed a soft tissue phlegmon while she was on treatment for meningitis: her CSF was consistently positive for *Exserohilum* by real-time PCR and she also had consistently elevated levels of BDG in CSF (Table 4). Overall, our data suggest that in most patients, the *Exserohilum* DNA in CSF declined below the reliable LOD level soon after treatment initiation. Detection of *Exserohilum* DNA may be an indication of relapse or lingering infection, but overall, BDG provide a better marker for monitoring response to treatment in most patients.

Our results demonstrate that, when used appropriately, real-time PCR can provide a valuable tool for confirming presence of fungal DNA in patients' specimens. However, this method is highly prone to contamination and should be used with caution and only in combination with clinical and epidemiological data. This outbreak provided a unique opportunity to evaluate different methods for molecular detection of filamentous fungi in clinical samples. Our results indicate that all three tested detection methods have limitations, and that using a comprehensive approach consisting of broad-spectrum PCR/sequencing, real-time PCR and BDG testing provided the best strategy for timely detection, identification and monitoring of fungal infection in this outbreak. Results obtained in this investigation will help to develop strategies for diagnosis and treatment of systemic mold infections in the future.

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Table 1. Reproducibility of real-time PCR assay

	DNA copy number[b]						
	10	10^2	10^3	10^4	10^5	10^6	10^7
CV[a] within assay (%)[c]	0.9	0.5	0.2	0.4	1.2	1.5	0.5
CV between assays (%)[d]	0.3	2.4	1.1	1.6	2.2	2.3	2.9

[a] CV Coefficient of Variation

[b] Ten-fold dilutions of linearized plasmid DNA; copies per reaction; dilution series thawed on 3 different days and assays performed in triplicate for each dilution

[c] Determined from three replicates within each assay.

[d] Determined from three independent assays performed on different days.

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Table 2. Comparison among real-time PCR, conventional PCR and culture (N=139)

Results by test performed	No. positive (%)
Real-Time PCR and Culture positive, conventional PCR negative	5 (4)
Real-Time PCR and conventional PCR positive, Culture negative	27 (19)
Positive by all 3 methods	14 (10)
All conventional PCR positive	41 (29)
All culture positive	19 (14)
All real-time PCR positive	65 (47)
Real-Time positive PCR only	19 (14)

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Table 3. Results of PCR and real-time PCR presented by patient and by sample.

Results for:	No. tested	No. positive (%) by PCR	No. positive (%) by real-time PCR
All case patients	471	114(24)	171(37)[a]
CSF	359	83(23)	138(38)[b]
Synovial fluids and Abscess aspirates	30	5(17)	6(21)[c]
Tissue fluids	82	26(33)	27(36)
All case specimens	898	123(14)	209(24)[a]
CSF	729	91(13)	174(24)[b]
Synovial fluids and Abscess aspirates	48	5(11)	7(15)[c]
Tissue fluids	121	27(22)	28(25)[d]

[a] 10 Specimen DNA were not tested since quantity was not sufficient

[b] 2 CSF DNAs were not tested

[c] 1 Abscess aspirate DNA was not tested

[d] 7 Tissue fluid DNAs were not tested; fresh and FFPE tissues were excluded

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Table 4. Test results, demographic and clinical information for patients with serially collected CSF.

Patient No.	Sex	Age, y	LP Date[a]	Culture	PCR Result	Real-Time PCR	Ct value	WBC/mL	BDG, pg/mL (± SD)[b]	Clinical Outcome
Patient 1	F	42	17-Oct-12	Neg	*Er*	*Er*	33	2507	N/P	
			7-Nov-12	Neg	Neg	Neg	0	1330	>500	Multiple strokes,
			10-Dec-12	Neg	Neg	Neg	0	104	N/P	deceased
			15-Jan-13	Neg	Neg	Neg	0	57	>500	
Patient 2	F	64	7-Oct-12	Neg	Neg	Ind	43	2576	>500	
			7-Nov-12	Neg	Neg	Neg	0	177	>500	Relapse meningitis,
			6-Feb-13	Neg	Neg	Neg	0	0	>500	on retreatment,
			16-May-13	Neg	*Cc*	Neg	0	513	>500	stable
			30-May-13	Neg	Neg	Neg	0	145	>500	
Patient 3[c]	M	80	4-Oct-12	Neg	Neg	*Er*	38	119	>500	Relapse meningitis,

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			17-Oct-12	Neg	Neg	Neg	0	63	>500	on treatment,
			30-Nov-12	Neg	Neg	Neg	0	9	488 ± 11	stable
			11-Jan-13	Neg	Neg	Neg	0	5	246 ± 75	
			11-Mar-13	Neg	*Er*	*Er*	36	2075	>500	
Patient 4	F	77	9-Oct-12	Neg	*Er*	*Er*	30	2550	>500	Relapse soft tissue
			8-Nov-12	Neg	Neg	*Er*	40	34	>500	phlegmon,
			5-Dec-12	Neg	Neg	*Er*	38	0	>500	on retreatment, stable
Patient 5	M	72	5-Oct-12	Neg	*Er*	*Er*	34	1989	>500	
			19-Dec-12	Neg	Neg	*Er*	39	5	>500	Completed treatment,
			13-Feb-13	Neg	Neg	Neg	0	2	387 ± 33	asymptomatic, stable
			3-Apr-13	Neg	Neg	Neg	0	7	218 ± 4	
Patient 6	M	69	4-Oct-12	Neg	Neg	*Er*	33	664	>500	Completed treatment,
			5-Dec-12	Neg	Neg	Neg	0	14	>500	asymptomatic,
			6-Mar-13	Neg	Neg	Neg	0	0	383 ± 32	stable

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Patient 7	F	64	11-Oct-12	Neg	*Er*	*Er*	30	3996	>500	
			25-Oct-12	Neg	Neg	Ind	41	43	N/P	
			2-Jan-13	Neg	Neg	Neg	0	7	>500	Completed treatment, asymptomatic, stable
			6-Feb-13	Neg	Neg	Ind	44	5	438 ± 23	
			8-Apr-13	Neg	Neg	Neg	0	5	408 ± 32	
			15-Jul-13	Neg	Neg	Neg	0	3	252 ± 103	
Patient 8	M	44	15-Oct-12	Neg	*Er*	*Er*	39	602	N/P	
			28-Nov-12	Neg	Neg	Neg	0	85	>500	Completed treatment, asymptomatic, stable
			4-Jan-13	Neg	Neg	Neg	0	7	>500	
			25-Feb-13	Neg	Neg	Neg	0	14	391 ± 14	
			1-Apr-13	Neg	Neg	Neg	0	5	457 ± 61	
			6-May-13	Neg	Neg	Neg	0	8	<32	
Patient 9	F	75	22-Oct-12	*Er*	*Er*	*Er*	31	N/P	N/P	Completed treatment, asymptomatic,
			12-Nov-12	Neg	Neg	Ind	44	3106	>500	

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			23-Nov-12	Neg	Neg	Ind	43	10	412 ± 3	stable
			1-Feb-13	Neg	Neg	Ind	42	14	238 ± 2	
			8-Mar-13	Neg	Neg	Neg	0	2	120 ± 25	
Patient 10	F	84	4-Oct-12	Neg	Neg	*Er*	34	2676	>500	
			21-Nov-12	Neg	Neg	Neg	0	66	>500	Continues on
			1-Mar-13	Neg	Neg	Neg	0	17	>500	treatment (8 months),
			5-Apr-13	*En*	Neg	Neg	0	157	427 ± 35	stable
			26-Apr-13	Neg	Neg	Neg	0	48	240 ± 14	
			28-May-13	Neg	Neg	Neg	0	43	<32	
Patient 11	M	65	4-Oct-12	Neg	*Er*	*Er*	28	1828	>500	
			2-Nov-12	Neg	Neg	Neg	0	71	235 ± 17	Completed treatment,
			9-Jan-13	Neg	Neg	Neg	0	2	<32	asymptomatic,
			7-Feb-13	Neg	Neg	Neg	0	0	41 ± 9	stable
			8-May-13	Neg	Neg	Neg	0	0	<32	

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Patient 12	F	58	29-Sep-12	*Cc*	Neg	Neg	0	9080	>500	
			22-Oct-12	Neg	Neg	Neg	0	173	N/P	Completed treatment, asymptomatic, stable
			21-Dec-12	Neg	Neg	Neg	0	2	112 ± 5	
			21-Mar-13	Neg	Neg	Neg	0	7	55	
Patient 13	M	77	8-Oct-12	Neg	*Er*	*Er*	30	1530	>500	
			9-Nov-12	Neg	Neg	Ind	42	6373	>500	Completed treatment, asymptomatic, stable
			3-Jan-13	Neg	Neg	Neg	0	65	>500	
			5-Feb-13	Neg	Neg	Neg	0	5	104 ± 0	
			5-Apr-13	Neg	Neg	Neg	0	17	129 ± 21	
			20-Apr-13	Neg	Neg	Neg	0	7	45 ± 0	
Patient 14	M	50	4-Oct-12	*Er*	*Er*	*Er*	32	830	>500	
			8-Nov-12	Neg	Neg	Neg	0	418	>500	Continues on
			18-Dec-12	Neg	Neg	Neg	0	121	>500	treatment (8 months),stable
			12-Mar-13	Neg	Neg	Neg	0	50	32 ± 61	

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			3-Jun-13	Neg	Neg	Neg	0	16	335	
			5-Aug-13	Neg	Neg	Neg	0	157	385 ± 25	
Patient 15	M	70	5-Oct-12	Neg	Neg	*Er*	39	2610	>500	
			8-Nov-12	Neg	Neg	Neg	0	7	297 ± 20	Completed treatment,
			12-Dec-12	Neg	Neg	Neg	0	2	241 ± 18	asymptomatic,
			6-Feb-13	Neg	Neg	Neg	0	0	69 ± 12	stable
			5-Apr-13	Neg	Neg	Neg	0	0	123 ± 2	
Patient 16	M	61	8-Oct-12	Neg	Neg	Ind	43	280	>500	
			12-Nov-12	Neg	Neg	Neg	0	52	385 ± 75	Completed treatment,
			5-Dec-12	Neg	Neg	Neg	0	0	41 ± 1	asymptomatic,
			4-Mar-13	Neg	Neg	Neg	0	0	<32	stable
			10-May-13	Neg	Neg	Neg	0	0	<32	
Patient 17	F	55	24-Oct-12	Neg	*Er*	*Er*	34	48	>500	Completed treatment,
			28-Nov-12	*En*	Neg	*Er*	38	954	>500	asymptomatic,

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			23-Jan-13	Neg	Neg	Ind	41	0	110 ± 4	stable
			1-Apr-13	Neg	Neg	Neg	0	2	50 ± 4	
Patient 18	F	92	15-Oct-12	Neg	Neg	*Er*	36	28	486 ± 124	
			19-Nov-12	Neg	Neg	Neg	0	2	426 ± 269	Completed treatment, asymptomatic,
			22-Jan-13	Neg	Neg	Ind	42	5	278 ± 11	stable
			1-May-13	Neg	Neg	Neg	0	2	266 ± 14	
Patient 19	M	16	29-Oct-12	Neg	*Er*	*Er*	30	710	450 ± 14	
			12-Dec-12	Neg	Neg	Neg	0	90	>500	Completed treatment, asymptomatic,
			21-Jan-13	Neg	Neg	Neg	0	12	171 ± 52	stable
			17-May-13	Neg	Neg	Neg	0	6	<32	
Patient 20	F	63	11-Oct-12	Neg	Neg	*Er*	39	777	>500	
			30-Oct-12	Neg	Neg	Neg	0	475	>500	Completed treatment, asymptomatic,
			18-Feb-13	Neg	Neg	Neg	0	33	280 ± 8	stable
			15-May-13	Neg	Neg	Neg	0	5	296 ± 18	

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Abbreviations: *Cc*, *Cladosporium cladosporioides*; *En*, *Epicoccum nigrum*; *Er*, *Exserohilum rostratum*; LP, lumbar puncture; N/P, not performed; WBC, white blood cell. Neg., Negative; Ind., Indeterminate

[a] Only LPs with culture, PCR, and/or BDG results are included. LP dates for Patients 1, 4, 8 and 9 were adjusted compared to previous report (6) based on clinical data review. CSF BDG positive cutoff was determined to be 138 pg/ml (6)

[b] Mean and standard deviation (SD) of the 3 readings.

[c] Ref 12

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