

# 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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*Exserohilum rostratum* was the major cause of the multistate outbreak of fungal meningitis linked to contaminated injections of methylprednisolone acetate produced by the New England Compounding Center. 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 the case patients. Here, we report the development and validation of a TaqMan real-time PCR assay for the 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 results positive for *E. rostratum* to 171 (37% of the 461 case patients with available specimens). Compared to fungal culture and the previous PCR assays, this real-time PCR assay was more sensitive. Of the 139 identical specimens from case patients tested by all three methods, 19 (14%) were positive by culture, 41 (29%) were positive by the conventional PCR assay, and 65 (47%) were positive by the real-time PCR assay. We also compared the utility of the real-time PCR assay with that of the previously described beta-D-glucan (BDG) detection assay for monitoring response to treatment in case patients with serially collected CSF. Only the incident CSF specimens from most of the case patients were positive by real-time PCR, while most of the subsequently collected specimens were negative, confirming our previous observations that the BDG assay was more appropriate than the real-time PCR assay for monitoring the response to treatment. Our results also demonstrate that the real-time PCR assay is extremely susceptible to contamination and its results should be used only in conjunction with clinical and epidemiological data.

Since September 2012, U.S. 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 *Exserohilum rostratum*, a plant pathogen that rarely causes disease in humans (4). By using culture, PCR, and histopathology, this fungus was identified in cerebrospinal fluid (CSF), synovial fluid, abscess aspirate, fresh tissue, and formalin-fixed, paraffin-embedded tissue samples from 153 case patients, as well as in 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 the case patients (3). Although the sensitivity of the PCR assay was considerably better than that of culture, which was able to confirm only 10% of the cases, a large percentage of the case patients remained without laboratory confirmation of infection. We also investigated the utility of the Fungitell assay for the detection of beta-D-glucan (BDG) in CSF for diagnosis and monitoring of the response to treatment (6). Using this assay and a stratified sample of CSF specimens, we demonstrated that approximately 45% of the CSF specimens from case patients with negative PCR assay results had elevated levels of BDG. Our results, as well as others, also demonstrated that consistently elevated levels of BDG in CSF may indicate 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 the development of a quantitative real-time PCR assay using TaqMan technology for detection of the internal transcribed spacer 2 (ITS2) ribosomal DNA region of *E. rostratum*. We report the results of sensitivity and specificity testing of this novel real-time PCR assay and compare its utility with that of previously reported conventional PCR and BDG detection assays.

Received 22 August 2014 Returned for modification 24 September 2014

Accepted 5 December 2014

Accepted manuscript posted online 17 December 2014

Citation Gade L, Grgurich DE, Kerkerling TM, Brandt ME, Litvintseva AP. 2015. 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. *J Clin Microbiol* 53:618–625. doi:10.1128/JCM.02443-14.

Editor: D. J. Diekema

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.02443-14>.

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## MATERIALS AND METHODS

**Human subjects.** This investigation was considered a public health emergency 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 the 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 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: 05212012@68, 06292012@26, or 08102012@51. These case definitions have been previously described (3, 11).

**Clinical specimens.** This study included specimens received between 2 October 2012 and 31 December 2013. A total of 898 specimens from 471 case patients were tested, including CSF, synovial fluid, abscess aspirate, and tissue fluid samples. 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 samples from 20 case patients whose specimens were previously tested for BDG detection were included to evaluate the utility of our real-time PCR assay for monitoring of the 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 with the QIAamp UltraSens virus kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions but with modifications described previously (5). The same DNA extractions were used for conventional and real-time PCRs. DNA was stored at  $-80^{\circ}\text{C}$  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, shipped on dry ice to the CDC, and kept at  $-80^{\circ}\text{C}$  until testing.

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

**Real-time PCR primers and probe.** To select specific real-time PCR primers and a probe, we used Mega 5.0 (13) to generate alignments of 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 with Primer 3 (<http://frodo.wi.mit.edu/>) and *E. rostratum* strain TA26-49 ITS DNA (NCBI accession number JF819166.1). The real-time PCR primers used were EXS 1F (5'-TTG TCT CTC CCC TTG TTG G-3') and EXS 1R (5'-CCG CCC CGT GGA TTG GAA-3'), and the probe was 5'-FAM-TTG GCA GCC GAC CTA CTG GTT TT-BHQ1-3', where FAM is 6-carboxy-fluorescein and BHQ is the Black Hole Quencher. The specificity of the primer and probe sequences selected was tested by BLAST by using the

NCBI nucleotide collection (nr/nt) database, and no significant similarity to any other reliably identified species of fungi was detected.

**Real-time PCR assay.** A 25- $\mu\text{l}$  reaction mixture contained 12.5  $\mu\text{l}$  of 2 $\times$  TaqMan universal master mix (Applied Bio-Systems, Gaithersburg, MD), 0.2  $\mu\text{M}$  each primer, 0.1  $\mu\text{M}$  TaqMan probe, and 5  $\mu\text{l}$  of template DNA. Reactions were performed with a Rotor-Gene real-time instrument (Qiagen RG 6000) under cycling conditions including an initial denaturation step at  $95^{\circ}\text{C}$  for 5 min, followed by 45 cycles of  $95^{\circ}\text{C}$  for 15 s,  $54^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  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 of the specimen DNAs were tested in duplicate.

**Analytical specificity of the real-time PCR primers and probe.** The analytical specificity of the primers and probe used was tested with *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*, and *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 assay, such as *C. cladosporioides*, *Cladosporium* sp., *E. nigrum*, *Alternaria* sp., and *Malassezia restricta*.

**Determination of LOD.** PCR products were cloned into the pCR 2.1 TA vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, linearized by digestion with HindIII (New England Bio Labs, Ipswich, MA) according to the manufacturer's instructions, purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and used to prepare 10-fold serial dilutions containing  $10^7$  to  $10^1$  plasmid copies per reaction mixture. A standard curve was generated with 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 limit of detection (LOD) of this method.

**Estimation of reproducibility.** Assay reproducibility was tested by comparing  $C_T$  values obtained on 3 different days with replicate 10-fold serial dilutions of the linearized plasmid DNA and measured by calculating the coefficient of variation (CV) of the mean  $C_T$  values between runs. Precision was evaluated for each dilution point by comparing variability among triplicates and measuring the CV of the mean  $C_T$  values within runs.

**Determination of diagnostic sensitivity and specificity.** To calculate diagnostic sensitivity, we compared the performance of the real-time PCR assay with that of culture and previously developed broad-spectrum and *E. rostratum*-specific conventional PCR assays with 139 previously described CSF samples from proven and probable cases with known PCR assay and culture results (5). Positive-control specimens were specimens that had been demonstrated positive for *E. rostratum* by conventional PCR assay ( $n = 41$ ) and/or culture ( $n = 19$ ). Diagnostic specificity was calculated by using 66 negative-control specimens from patients who were not considered case patients 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 DNA extraction procedures.

## RESULTS

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

**LOD and amplification efficiency.** The LOD was approximately 10 copies per reaction, which corresponded to a  $C_T$  value of

TABLE 1 Reproducibility of real-time PCR assay results

Comparison	CV (%) with DNA copy no. <sup>a</sup> of:						
	10	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>
Within assay <sup>b</sup>	0.9	0.5	0.2	0.4	1.2	1.5	0.5
Between assays <sup>c</sup>	0.3	2.4	1.1	1.6	2.2	2.3	2.9

<sup>a</sup> Tenfold dilutions of linearized plasmid DNA were used, numbers of copies per reaction mixture are shown, dilution series were thawed on 3 different days, and assays were performed in triplicate for each dilution.

<sup>b</sup> Determined from three replicates within each assay.

<sup>c</sup> Determined from three independent assays performed on different days.

40 ± 0.3. Strong linear correlations ( $r^2$ , >0.99) were obtained between  $C_T$  values and target copy numbers over a 7-log range (10 to 10<sup>7</sup> copies per reaction) with 98% amplification efficiency (see Fig. S1 in the supplemental material).

**Reproducibility.** Assay reproducibility was tested by using replicate 10-fold serial dilutions of the linearized plasmid DNA, and intra- and interassay variability was evaluated for each dilution point in triplicate on 3 different days. The CVs of the mean  $C_T$  values within and between runs were 0.20 to 1.5% and 0.2 to 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 (47%) of these specimens were positive by real-time PCR, 41 (29%) were positive by conventional PCR assays, and 19 (14%) were positive by culture (5). All of the specimens positive by conventional PCR assays and/or culture were also positive by real-time PCR assay. Furthermore, five specimens that were positive by culture but negative by conventional PCR assays in previous testing also were positive by real-time PCR assay. In addition, 19 specimens that were negative by both conventional PCR assays and culture were positive by real-time PCR assay (Table 2).

**Diagnostic specificity of the real-time PCR assay.** 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 ( $C_T$  value 0); however, a low level of *E. rostratum* DNA ( $C_T$  value of 42 or 43) was detected in 7 (11% of 61) samples (data not shown). Furthermore, we tested 66 CSF samples from patients not considered case patients in the outbreak. For 56 (85% of 66) of these samples, no amplification was observed after 45 cycles of PCR ( $C_T$  value of 0); however, low levels of *E. rostratum* DNA were detected ( $C_T$  values of 41

TABLE 2 Comparison of real-time and conventional PCR assay and culture results obtained with 139 samples

Result(s) by test(s) 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 PCR positive only	19 (14)

TABLE 3 Conventional and real-time PCR assays results presented by patient and by sample

Results for:	No. of samples tested	No. positive (%) by:	
		Conventional PCR	Real-time PCR
All case patients	471	114 (24)	171 (37) <sup>a</sup>
CSF samples	359	83 (23)	138 (38) <sup>b</sup>
Synovial fluids, abscess aspirates	30	5 (17)	6 (21) <sup>c</sup>
Tissue fluid samples	82	26 (33)	27 (36)
All case specimens	898	123 (14)	209 (24) <sup>a</sup>
CSF samples	729	91 (13)	174 (24) <sup>b</sup>
Synovial fluids, abscess aspirates	48	5 (11)	7 (15) <sup>c</sup>
Tissue fluid samples	121	27 (22)	28 (25) <sup>d</sup>

<sup>a</sup> Ten specimen DNA samples were not tested because the quantity was not sufficient.

<sup>b</sup> Two CSF DNA samples were not tested.

<sup>c</sup> One abscess aspirate DNA sample was not tested.

<sup>d</sup> Seven tissue fluid DNA samples were not tested; fresh and formalin-fixed, paraffin-embedded tissues were excluded.

to 45) in 10 (15% of 66) of these samples (data not shown). On the basis of these observations,  $C_T$  values of ≤40 were considered positive,  $C_T$  values of 41 to 45 were considered indeterminate, and a  $C_T$  value of 0 (>45) was considered a negative result.

**Results of real-time PCR assay.** Between October 2012 and December 2013, 898 CSF, synovial fluid, abscess aspirate, and tissue fluid samples 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 the real-time PCR assay because of an insufficient specimen or DNA quantity.

A total of 209 samples (24% of the samples tested) collected from 171 case patients (37% of the 461 case patients) were found to be positive for *E. rostratum* DNA by real-time PCR assay (Table 3). Positive results were obtained for 174 CSF samples from 138 case patients (38% of the 359 case patients with CSF samples), 7 synovial or abscess fluid samples from 6 case patients (21% of the 30 case patients with synovial fluid specimens), and 28 tissue fluid samples from 27 case patients (33% of the 82 case patients with tissue fluid specimens) (Table 3). The median  $C_T$  value for the positive samples was 37.6. Most of the real-time PCR assay-positive specimens (193 [92%] of 209 positive specimens) were received in October to December of 2012.

**Testing of serial CSF samples.** Results obtained by conventional PCR assay, real-time PCR assay, and measurement of BDG levels in CSF were compared among 20 case patients from whom samples were serially collected as described previously (6) (Table 4). Both conventional and real-time PCR assays were more likely to detect fungal DNA in the incident CSF samples than in samples serially collected later (Table 4). Specifically, incident CSF samples from 11 case patients were positive by conventional PCR assay and 18 were positive by real-time PCR assay. In addition, the real-time PCR assay detected *E. rostratum* DNA in two subsequent CSF specimens collected from patients 5 and 17 approximately 1 month after the first samples were collected. Furthermore, both the conventional and real-time PCR assays 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

TABLE 4 Test results and demographic and clinical information for patients with serially collected CSF samples<sup>a</sup>

Patient no., sex, <sup>b</sup> age (yr), and LP <sup>c</sup> date (day, mo, yr)	Result of:			<i>C<sub>T</sub></i>	No. of WBC <sup>d</sup> /ml	Mean BDG level (pg/ml) ± SD <sup>e</sup>	Clinical outcome
	Culture	PCR	Real-time PCR				
1, F, 42							
17 Oct. 2012	Neg <sup>f</sup>	<i>E. rostratum</i>	<i>E. rostratum</i>	33	2,507	NP <sup>g</sup>	Multiple strokes, deceased
7 Nov. 2012	Neg	Neg	Neg	0	1,330	>500	
10 Dec. 2012	Neg	Neg	Neg	0	104	NP	
15 Jan. 2013	Neg	Neg	Neg	0	57	>500	
2, F, 64							
7 Oct. 2012	Neg	Neg	Ind <sup>h</sup>	43	2,576	>500	Relapse meningitis, on retreatment, stable
7 Nov. 2012	Neg	Neg	Neg	0	177	>500	
6 Feb. 2013	Neg	Neg	Neg	0	0	>500	
16 May 2013	Neg	<i>C. cladosporioides</i>	Neg	0	513	>500	
30 May 2013	Neg	Neg	Neg	0	145	>500	
3, <sup>i</sup> M, 80							
4 Oct. 2012	Neg	Neg	<i>E. rostratum</i>	38	119	>500	Relapse meningitis, on treatment, stable
17 Oct. 2012	Neg	Neg	Neg	0	63	>500	
30 Nov. 2012	Neg	Neg	Neg	0	9	488 ± 11	
11 Jan. 2013	Neg	Neg	Neg	0	5	246 ± 75	
11 Mar. 2013	Neg	<i>E. rostratum</i>	<i>E. rostratum</i>	36	2,075	>500	
4, F, 77							
9 Oct. 2012	Neg	<i>E. rostratum</i>	<i>E. rostratum</i>	30	2,550	>500	Relapse soft tissue phlegmon, on retreatment, stable
8 Nov. 2012	Neg	Neg	<i>E. rostratum</i>	40	34	>500	
5 Dec. 2012	Neg	Neg	<i>E. rostratum</i>	38	0	>500	
5, M, 72							
5 Oct. 2012	Neg	<i>E. rostratum</i>	<i>E. rostratum</i>	34	1,989	>500	Completed treatment, asymptomatic, stable
19 Dec. 2012	Neg	Neg	<i>E. rostratum</i>	39	5	>500	
13 Feb. 2013	Neg	Neg	Neg	0	2	387 ± 33	
3 Apr. 2013	Neg	Neg	Neg	0	7	218 ± 4	
6, M, 69							
4 Oct. 2012	Neg	Neg	<i>E. rostratum</i>	33	664	>500	Completed treatment, asymptomatic, stable
5 Dec. 2012	Neg	Neg	Neg	0	14	>500	
6 Mar. 2013	Neg	Neg	Neg	0	0	383 ± 32	
7, F, 64							
11 Oct. 2012	Neg	<i>E. rostratum</i>	<i>E. rostratum</i>	30	3,996	>500	Completed treatment, asymptomatic, stable
25 Oct. 2012	Neg	Neg	Ind	41	43	NP	
2 Jan. 2013	Neg	Neg	Neg	0	7	>500	
6 Feb. 2013	Neg	Neg	Ind	44	5	438 ± 23	
8 Apr. 2013	Neg	Neg	Neg	0	5	408 ± 32	
15 Jul. 2013	Neg	Neg	Neg	0	3	252 ± 103	
8, M, 44							
15 Oct. 2012	Neg	<i>E. rostratum</i>	<i>E. rostratum</i>	39	602	NP	Completed treatment, asymptomatic, stable
28 Nov. 2012	Neg	Neg	Neg	0	85	>500	
4 Jan. 2013	Neg	Neg	Neg	0	7	>500	
25 Feb. 2013	Neg	Neg	Neg	0	14	391 ± 14	
1 Apr. 2013	Neg	Neg	Neg	0	5	457 ± 61	
6 May 2013	Neg	Neg	Neg	0	8	<32	
9, F, 75							
22 Oct. 2012	<i>E. rostratum</i>	<i>E. rostratum</i>	<i>E. rostratum</i>	31	NP	NP	Completed treatment, asymptomatic, stable
12 Nov. 2012	Neg	Neg	Ind	44	3,106	>500	
23 Nov. 2012	Neg	Neg	Ind	43	10	412 ± 3	
1 Feb. 2013	Neg	Neg	Ind	42	14	238 ± 2	
8 Mar. 2013	Neg	Neg	Neg	0	2	120 ± 25	

(Continued on following page)

TABLE 4 (Continued)

Patient no., sex, <sup>b</sup> age (yr), and LP <sup>c</sup> date (day, mo, yr)	Result of:			<i>C<sub>T</sub></i>	No. of WBC <sup>d</sup> /ml	Mean BDG level (pg/ml) ± SD <sup>e</sup>	Clinical outcome
	Culture	PCR	Real-time PCR				
10, F, 84							
4 Oct. 2012	Neg	Neg	<i>E. rostratum</i>	34	2,676	>500	Continues on treatment (8 mo), stable
21 Nov. 2012	Neg	Neg	Neg	0	66	>500	
1 Mar. 2013	Neg	Neg	Neg	0	17	>500	
5 Apr. 2013	<i>E. nigrum</i>	Neg	Neg	0	157	427 ± 35	
26 Apr. 2013	Neg	Neg	Neg	0	48	240 ± 14	
28 May 2013	Neg	Neg	Neg	0	43	<32	
11, M, 65							
4 Oct. 2012	Neg	<i>E. rostratum</i>	<i>E. rostratum</i>	28	1,828	>500	Completed treatment, asymptomatic, stable
2 Nov. 2012	Neg	Neg	Neg	0	71	235 ± 17	
9 Jan. 2013	Neg	Neg	Neg	0	2	<32	
7 Feb. 2013	Neg	Neg	Neg	0	0	41 ± 9	
8 May 2013	Neg	Neg	Neg	0	0	<32	
12, F, 58							
29 Sep. 2012	<i>C. cladosporioides</i>	Neg	Neg	0	9,080	>500	Completed treatment, asymptomatic, stable
22 Oct. 2012	Neg	Neg	Neg	0	173	NP	
21 Dec. 2012	Neg	Neg	Neg	0	2	112 ± 5	
21 Mar. 2013	Neg	Neg	Neg	0	7	55	
13, M, 77							
8 Oct. 2012	Neg	<i>E. rostratum</i>	<i>E. rostratum</i>	30	1,530	>500	Completed treatment, asymptomatic, stable
9 Nov. 2012	Neg	Neg	Ind	42	6,373	>500	
3 Jan. 2013	Neg	Neg	Neg	0	65	>500	
5 Feb. 2013	Neg	Neg	Neg	0	5	104 ± 0	
5 Apr. 2013	Neg	Neg	Neg	0	17	129 ± 21	
20 Apr. 2013	Neg	Neg	Neg	0	7	45 ± 0	
14, M, 50							
4 Oct. 2012	<i>E. rostratum</i>	<i>E. rostratum</i>	<i>E. rostratum</i>	32	830	>500	Continues on treatment (8 mo),stable
8 Nov. 2012	Neg	Neg	Neg	0	418	>500	
18 Dec. 2012	Neg	Neg	Neg	0	121	>500	
12 Mar. 2013	Neg	Neg	Neg	0	50	32 ± 61	
3 Jun. 2013	Neg	Neg	Neg	0	16	335	
5 Aug. 2013	Neg	Neg	Neg	0	157	385 ± 25	
15, M, 70							
5 Oct. 2012	Neg	Neg	<i>E. rostratum</i>	39	2,610	>500	Completed treatment, asymptomatic, stable
8 Nov. 2012	Neg	Neg	Neg	0	7	297 ± 20	
12 Dec. 2012	Neg	Neg	Neg	0	2	241 ± 18	
6 Feb. 2013	Neg	Neg	Neg	0	0	69 ± 12	
5 Apr. 2013	Neg	Neg	Neg	0	0	123 ± 2	
16, M, 61							
8 Oct. 2012	Neg	Neg	Ind	43	280	>500	Completed treatment, asymptomatic, stable
12 Nov. 2012	Neg	Neg	Neg	0	52	385 ± 75	
5 Dec. 2012	Neg	Neg	Neg	0	0	41 ± 1	
4 Mar. 2013	Neg	Neg	Neg	0	0	<32	
10 May 2013	Neg	Neg	Neg	0	0	<32	
17, F, 55							
24 Oct. 2012	Neg	<i>E. rostratum</i>	<i>E. rostratum</i>	34	48	>500	Completed treatment, asymptomatic, stable
28 Nov. 2012	<i>E. nigrum</i>	Neg	<i>E. rostratum</i>	38	954	>500	
23 Jan. 2013	Neg	Neg	Ind	41	0	110 ± 4	
1 Apr. 2013	Neg	Neg	Neg	0	2	50 ± 4	

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TABLE 4 (Continued)

Patient no., sex, <sup>b</sup> age (yr), and LP <sup>c</sup> date (day, mo, yr)	Result of:			<i>C<sub>T</sub></i>	No. of WBC <sup>d</sup> /ml	Mean BDG level (pg/ml) ± SD <sup>e</sup>	Clinical outcome
	Culture	PCR	Real-time PCR				
18, F, 92							
15 Oct. 2012	Neg	Neg	<i>E. rostratum</i>	36	28	486 ± 20,124	Completed treatment, asymptomatic, stable
19 Nov. 2012	Neg	Neg	Neg	0	2	426 ± 269	
22 Jan. 2013	Neg	Neg	Ind	42	5	278 ± 11	
1 May 2013	Neg	Neg	Neg	0	2	266 ± 14	
19, M, 16							
29 Oct. 2012	Neg	<i>E. rostratum</i>	<i>E. rostratum</i>	30	710	450 ± 14	Completed treatment, asymptomatic, stable
12 Dec. 2012	Neg	Neg	Neg	0	90	>500	
21 Jan. 2013	Neg	Neg	Neg	0	12	171 ± 52	
17 May 2013	Neg	Neg	Neg	0	6	<32	
20, F, 63							
11 Oct. 2012	Neg	Neg	<i>E. rostratum</i>	39	777	>500	Completed treatment, asymptomatic, stable
30 Oct. 2012	Neg	Neg	Neg	0	475	>500	
18 Feb. 2013	Neg	Neg	Neg	0	33	280 ± 8	
15 May 2013	Neg	Neg	Neg	0	5	296 ± 18	

<sup>a</sup> Only lumbar punctures with culture, PCR assay, and/or BDG results are included.

<sup>b</sup> F, female; M, male.

<sup>c</sup> LP, lumbar puncture. Lumbar puncture dates for patients 1, 4, 8, and 9 were adjusted compared to a previous report (6) based on clinical data review.

<sup>d</sup> WBC, white blood cells.

<sup>e</sup> Mean values and standard deviations of three readings are shown. The CSF BDG positive cutoff was determined to be 138 pg/ml (6).

<sup>f</sup> Neg, negative.

<sup>g</sup> NP, not performed.

<sup>h</sup> Ind, indeterminate.

<sup>i</sup> Reference 12.

from patient 4, who developed a soft tissue phlegmon, were positive by the real-time PCR assay, while only the incident CSF from this patient was positive by the conventional PCR assay. All of the CSF samples positive by the real-time PCR assay also had elevated BDG levels (Table 4). Furthermore, in most of the case patients, elevated BDG levels persisted for an additional 3 to 5 months when the real-time PCR assay results were indeterminate or negative (Table 4).

## DISCUSSION

The 2012 multistate outbreak of fungal meningitis and other infections has been the largest iatrogenic outbreak in U.S. history and is still affecting the lives of hundreds of people. By 23 October 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 the case patients from whom specimens were submitted to the CDC) by histopathology, PCR assay, or culture (3, 14). In addition, with input from the Infectious Diseases Society of America and an advisory group of experts in clinical mycology, the CDC developed treatment guidelines for physicians treating patients in the outbreak (11, 16). Although the recommended clinical management of 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 the case patients for whom specimens were submitted to the CDC) (5). Here, we report the development of a novel real-time PCR assay for detection of *Exserohilum* DNA in case patients in the outbreak and provide

laboratory confirmation of *Exserohilum* infection in 57 additional case patients.

The greater sensitivity of the real-time PCR assay than the conventional PCR assay was not surprising, since the real-time PCR assay 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 assay results, as all of the samples positive by the conventional PCR assay were also positive by the real-time PCR assay. In addition, all of the samples positive by culture were also positive by the real-time PCR assay, and five samples that were positive by culture but negative by conventional PCR assay were positive by the real-time PCR assay.

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 DNAs. 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 the case patients tested. Second, extracting fungal DNA from patient specimens remains the main challenge for this molecular detection method, since PCR assay sensitivity depends on the efficiency of DNA extraction. Here, we used a DNA extraction method that relies on the 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 contam-

ination. Even minuscule 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 the contamination and cross-contamination that occur during sample collection and handling, we used the following procedures. First, prior to their use in the real-time PCR assay, we tested all of the reagents for the presence of fungal DNA. 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 to process patient specimens. When these control water samples were tested by the real-time PCR assay, a low level of amplification ( $C_T$  value of 42 or 43) was detected in 7 (11%) of 61 samples, indicating cross-contamination during extraction.

Recently, there has been an increased interest in using PCR-based methods for the diagnosis of fungal infections in this outbreak and in other patient populations (9, 10, 17, 18). Our data suggest that PCR assay results need to be carefully scrutinized and should be considered only in combination with clinical and epidemiological data. False-positive PCR assay results caused by contamination during sample collection and processing have been reported by others (19–21), and our results support these observations. Reagent impurity and contamination during sample collection from skin flora and/or the 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 assay in CSF and other body fluids and tissues (14). Although some of these agents, such as *C. cladosporioides*, have been implicated in this outbreak, others, such as common wheat (*Triticum* sp.) or skin flora *M. restricta*, which we PCR amplified from the CSF of several case patients (data not shown), were unquestionably contaminants. Recently, Zhao et al. used a real-time PCR assay to detect the presence of fungal DNA in the blood of patients exposed 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 *A. fumigatus* was isolated from the index case patient (22), extensive screening of more than 1,000 specimens from more than 500 case patients in this outbreak by broad-spectrum fungal PCR assay in our laboratory (14), as well as independent screening with *Aspergillus*-specific PCR assay at a Virginia hospital (12), failed to detect any evidence of *A. fumigatus* DNA in any patient other than the index case patient (3). Overall, our experience with PCR and real-time PCR assays 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 the response to treatment in serially collected CSF samples. Here, we investigated the utility of a real-time PCR assay for monitoring of the patient response to treatment. Our results demon-

strated that although the real-time PCR assay was able to detect *Exserohilum* DNA in a larger number of case patients with serially collected CSF than the conventional PCR assay, in most patients, only CSF samples collected during the early stages of disease were positive by this test. Specifically, *Exserohilum* DNA was detected by real-time PCR assay in 17 (85%) of 20 case patients with serially collected samples. In 13 (76%) of those patients, only the incident specimens were positive; in 2 (12%, patient 5 and 17), both the incident samples and subsequent samples collected 1 month after the initial diagnosis were positive; and in 1 (6%, patient 3) who relapsed with meningitis, both the incident and relapse CSF samples were positive by real-time PCR assay (Table 4). The only exception was patient 4, who developed a soft tissue phlegmon while she was being treated for meningitis; her CSF was consistently positive for *Exserohilum* by real-time PCR assay, and she also had consistently elevated levels of BDG in her CSF (Table 4). Overall, our data suggest that, in most patients, the *Exserohilum* DNA level in CSF declined below the reliable LOD soon after treatment initiation. Detection of *Exserohilum* DNA may be an indication of relapse or lingering infection, but overall, BDG provides a better marker for monitoring the response to treatment in most patients.

Our results demonstrate that, when used appropriately, a real-time PCR assay can provide a valuable tool for confirming the 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 of the detection methods tested have limitations and that using a comprehensive approach consisting of a broad-spectrum PCR assay with sequencing, a real-time PCR assay, and BDG testing provided the best strategy for the timely detection, identification, and monitoring of fungal infection in this outbreak. Results obtained in this investigation will help to develop strategies for the diagnosis and treatment of systemic mold infections in the future.

## ACKNOWLEDGMENTS

We thank all of the participants in the Multistate Fungal Infection Outbreak Response Team, especially state and local health department officials, clinicians, and health care professionals, who provided clinical samples and data for this investigation. We thank all of the members of the CDC Mycotic Diseases Branch and CDC Emergency Response volunteers for their help with receiving, processing, and cataloging clinical samples, especially Christina Scheel, Cau Pham, Mark Lindsley, Nina Grossman, Joyce Peterson, Carol Bolden, Ngoc Le, Shirley McClinton, Randy Kuykendall, Steve Hurst, Kizee Etienne, Noelle Bessette, Grant Williams, Paris Paul, Sarah Yi, Laura Dickmeyer, and Jessica Halpin. We thank Michael Palmieri of the FDA for providing vial isolates and Deanna Sutton, Michael Rinaldi, and Michael McGinnis for providing control isolates for this study. We thank Rachel Smith, Benjamin Park, and Tom Chiller for critically reading the manuscript.

The use of product names in this report does not imply their endorsement by the U.S. Department of Health and Human Services. The finding and conclusions in this article are those of the authors and do not necessarily represent the views of the CDC.

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