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

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ABSTRACT

25 *Exserohilum rostratum* was the major cause of the multistate outbreak of fungal
26 meningitis linked to contaminated injections of methylprednisolone acetate (MPA)
27 produced by the New England Compounding Center (NECC). Previously, we developed
28 a fungal DNA extraction procedure and broad range and *E. rostratum*- specific PCR
29 assays, and confirmed the presence of fungal DNA in 28% of case-patients. Here we
30 report the development and validation of a TaqMan[®] real-time PCR assay for detection
31 of *E. rostratum* in body fluids, which we used to confirm infections in 57 additional case-
32 patients, bringing the total number of case-patients with PCR-positive results for *E.*
33 *rostratum* to 171 (37% of 461 case-patients with available specimens). Compared to
34 fungal culture and the previous PCR assays, this real-time PCR assay was more
35 sensitive: of 139 case-patients with identical specimens tested by all three methods, 19
36 (14%) specimens were positive by culture, 41 (29%) positive by the conventional PCR,
37 and 65 (47%) positive by the real-time PCR. We also compared utility of the real-time
38 PCR with the previously described beta-D-glucan (BDG) detection assay for monitoring
39 response to treatment in case-patients with serially collected CSF. In most case-
40 patients, only the incident CSF specimens were positive by real-time PCR while most
41 subsequently collected specimens were negative, confirming our previous observations
42 that BDG assay was more appropriate than real-time PCR for monitoring response to
43 treatment. Our results also demonstrate that real-time PCR is extremely susceptible to
44 contamination and its results should only be used in conjunction with clinical and
45 epidemiological data.

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INTRODUCTION

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

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

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

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MATERIALS AND METHODS

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

87 **Case definitions.** A proven case was defined as a probable case with evidence (by
88 culture, histopathology, or molecular assay) of a fungal pathogen associated with the
89 clinical syndrome. A probable case was defined as development of any of the following:
90 meningitis of unknown etiology following epidural or paraspinal injection after 21 May
91 2012; posterior circulation stroke without a cardioembolic source and without
92 documentation of a normal CSF profile following epidural or paraspinal injection after 21

93 May 2012; osteomyelitis, abscess, or other infection (e.g., soft tissue infection) of
94 unknown etiology in the spinal or paraspinal structures at or near the site of injection
95 following epidural or paraspinal injection after 21 May 2012; or osteomyelitis or
96 worsening inflammatory arthritis of a peripheral joint (e.g., knee, shoulder, or ankle) of
97 unknown etiology diagnosed following joint injection after 21 May 2012 in a person who
98 received an injection with preservative-free MPA that definitely or likely came from one
99 of the following three lots produced by the New England Compounding Center (NECC):
100 05212012@68, 06292012@26, 08102012@51. These case definitions have been
101 previously described (3, 11).

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

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

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

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

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

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

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

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

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

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

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

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RESULTS

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

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

194 **Reproducibility.** Assay reproducibility was tested by using replicate 10-fold serial
195 dilutions of the linearized plasmid DNA and intra- and inter assay variability was
196 evaluated for each dilution point in triplicate on three different days. The coefficient of
197 variation (CV) of the mean Ct values within and between runs was 0.20%-1.5% and
198 0.2%-2.9%, respectively (Table 1).

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

207 19 specimens that were negative by both conventional PCR and culture were positive
208 with real-time PCR (Table 2).

209 **Diagnostic specificity of the real-time PCR.** To determine the specificity of the assay,
210 we tested 61 control water samples that were subjected to the same DNA extraction
211 procedure as the patient specimens (5): for 54 (89% of 61) of these samples, no
212 amplification was observed after 45 cycles (Ct value 0); however, a low level of *E.*
213 *rostratum* DNA (Ct value 42-43) was detected in 7(11% of 61) samples (data not
214 shown). Furthermore, we tested 66 CSF samples from patients not considered cases in
215 the outbreak. For 56 (85% of 66) of these samples, no amplification was observed after
216 45 cycles of PCR (Ct value 0); however, low levels of *E. rostratum* DNA were detected
217 (Ct value 41-45) in 10 (15% of 66) of these samples (data not shown). Based on these
218 observations, Ct values ≤ 40 were considered positive, Ct values of 41- 45 were
219 considered indeterminate, and Ct values of 0 (>45) were considered a negative result.

220 **Results of real-time PCR Assay.** Between October 2012 and December 2013, 898
221 CSFs, synovial fluids, abscess aspirates and tissue fluids from 471 case patients were
222 tested by the broad-range and *E. rostratum*-specific conventional PCR assays and DNA
223 sequencing (5). Of those, 888 specimens from 461 case-patients were available for
224 real-time PCR testing. Ten samples tested previously by the conventional PCR assay
225 were not tested by real-time PCR because of an insufficient quantity of specimen/DNA.
226 A total of 209 samples (24% of all tested samples) collected from 171 case-patients
227 (37% of 461 case-patients) were found positive for *E. rostratum* DNA using real-time
228 PCR (Table 3). Positive results were obtained for 174 CSF from 138 case-patients
229 (38% of 359 case-patients with CSF samples), 7 synovial/abscess fluids from 6 case-
230 patients (21% of 30 case-patients with synovial specimens) and 28 tissue fluids from 27

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

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

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DISCUSSION

255 The 2012 multistate outbreak of fungal meningitis and other infections has been
256 the largest iatrogenic outbreak in US history, and is still affecting the lives of hundreds
257 of people. By October 23, 2013, in 20 states 752 cases were identified, resulting in 64
258 deaths. CDC laboratories were able to confirm fungal infections in 173 case-patients
259 (33% of case-patients from whom specimens were submitted to CDC) using
260 histopathology, PCR or culture (3, 14). In addition, with input from the Infectious
261 Diseases Society of America and an advisory group of experts in clinical mycology,
262 CDC developed treatment guidelines for physicians treating patients from the outbreak
263 (11, 16). Although the recommended clinical management for both confirmed and
264 probable cases in the outbreak was the same, there was a need to develop a more
265 sensitive diagnostic test to better understand the etiology of this outbreak. Our
266 previously developed conventional PCR assays were able to detect fungal DNA in 114
267 case-patients with available body fluid specimens (25% of case-patients for whom
268 specimens were submitted to CDC)(5). Here, we report development of a novel real-
269 time PCR assay for detection of *Exserohilum* DNA in case-patients from the outbreak
270 and provide laboratory confirmation of *Exserohilum* infection in 57 additional case-
271 patients.

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

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

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

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

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

307 Recently, there has been an increased interest in using PCR-based methods for
308 diagnosis of fungal infections from this outbreak and in other patient populations (9, 10,
309 17, 18). Our data suggest that PCR results need to be carefully scrutinized and should
310 only be considered in combination with clinical and epidemiological data. False positive
311 PCR results caused by contamination during sample collection and processing have
312 been reported by others (19-21) and our results support these observations. Reagent
313 impurity, contamination during sample collection from skin flora and/or environment, as
314 well as cross-contamination during DNA extraction and processing cannot be
315 completely avoided, and therefore, amplification of fungal DNA from a biological sample
316 does not always imply infection. For example, in this outbreak several other
317 environmental fungi have been detected by PCR in CSF and other body fluids and
318 tissues (14). Although some of these agents, such as *Cladosporium cladosporioides*,
319 have been implicated in this outbreak, others, such as common wheat (*Triticum sp.*) or
320 skin flora *Malassezia restricta*, which we PCR-amplified from CSF of several case-
321 patients (data not shown), were unquestionably contaminants. Recently, Zhao et al
322 used real-time PCR to detect the presence of fungal DNA in blood of patients exposed

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

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

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

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

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References

- 384 1. **Centers for Disease Control and Prevention.** 2012. Multistate outbreak of
385 fungal infection associated with injection of methylprednisolone acetate solution
386 from a single compounding pharmacy - United States, 2012. *Morb Mortal Wkly*
387 *Rep* **61**:839-842.
- 388 2. **Centers for Disease Control and Prevention.** 2013. Spinal and paraspinal
389 infections associated with contaminated methylprednisolone acetate injections -
390 Michigan, 2012-2013. *Morb Mortal Wkly Rep* **62**:377-381.

- 391 3. **Smith RM, Schaefer MK, Kainer MA, Wise M, Finks J, Duwve J, Fontaine E,**
392 **Chu A, Carothers B, Reilly A, Fiedler J, Wiese AD, Feaster C, Gibson L,**
393 **Griese S, Purfield A, Cleveland AA, Benedict K, Harris JR, Brandt ME, Blau**
394 **D, Jernigan J, Weber JT, Park BJ, Multistate Fungal Infection Outbreak**
395 **Response T.** 2013. Fungal infections associated with contaminated
396 methylprednisolone injections. *N Engl J Med* **369**:1598-1609.
- 397 4. **Litvintseva AP, Hurst S, Gade L, Frace MA, Hilsabeck R, Schupp JM, Gillece**
398 **JD, Roe C, Smith D, Keim P, Lockhart SR, Changayil S, Weil MR,**
399 **MacCannell DR, Brandt ME, Engelthaler DM.** 2014. Whole genome analysis of
400 *Exserohilum rostratum* from the outbreak of fungal meningitis and other
401 infections. *J Clin Microbiol* **52(9)**:3216-22.
- 402 5. **Gade L, Scheel CM, Pham CD, Lindsley MD, Iqbal N, Cleveland AA, Whitney**
403 **AM, Lockhart SR, Brandt ME, Litvintseva AP.** 2013. Detection of fungal DNA
404 in human body fluids and tissues during a multistate outbreak of fungal
405 meningitis and other infections. *Eukaryot Cell* **12**:677-683.
- 406 6. **Litvintseva AP, Lindsley MD, Gade L, Smith R, Chiller T, Lyons JL, Thakur**
407 **KT, Zhang SX, Grgurich DE, Kerkering TM, Brandt ME, Park BJ.** 2014. Utility
408 of (1-3)-beta-D-glucan testing for diagnostics and monitoring response to
409 treatment during the multistate outbreak of fungal meningitis and other infections.
410 *Clin Infect Dis* **58**:622-630.
- 411 7. **Nelson G, Fermo O, Thakur K, Felton E, Bang J, Wilson L, Rhee S, Llinas R,**
412 **Johnson K, Sullivan D.** 2014. Resolution of a fungal mycotic aneurysm after a
413 contaminated steroid injection: a case report. *BMC Res Note* **7**:327.

- 414 8. **Lyons JL, Roos KL, Marr KA, Neumann H, Trivedi JB, Kimbrough DJ,**
415 **Steiner L, Thakur KT, Harrison DM, Zhang SX.** 2013. Cerebrospinal fluid (1,
416 3)- β -d-glucan detection as an aid for diagnosis of iatrogenic fungal meningitis. J
417 Clin Microbiol **51**:1285-1287.
- 418 9. **Zhao Y, Petraitiene R, Walsh TJ, Perlin DS.** 2013. A real-time PCR assay for
419 rapid detection and quantification of *Exserohilum rostratum*, a causative
420 pathogen of fungal meningitis associated with injection of contaminated
421 methylprednisolone. J Clin Microbiol **51**:1034-1036.
- 422 10. **Zhao Y, Armeanu E, DiVerniero R, Lewis TA, Dobson RC, Kontoyiannis DP,**
423 **Roilides E, Walsh TJ, Perlin DS.** 2014. Fungal DNA Detected in Blood Samples
424 of Patients Who Received Contaminated Methylprednisolone Injections Reveals
425 Increased Complexity of Causative Agents. J Clinical Microbiol **52**:2212-2215.
- 426 11. **Chiller TM, Roy M, Nguyen D, Guh A, Malani AN, Latham R, Peglow S,**
427 **Kerkering T, Kaufman D, McFadden J, Collins J, Kainer M, Duwve J, Trump**
428 **D, Blackmore C, Tan C, Cleveland AA, MacCannell T, Muehlenbachs A, Zaki**
429 **SR, Brandt ME, Jernigan JA, Multistate Fungal Infection Clinical**
430 **Investigation T.** 2013. Clinical findings for fungal infections caused by
431 methylprednisolone injections. N Engl J Med **369**:1610-1619.
- 432 12. **Kerkering TM, Grifasi ML, Baffoe-Bonnie AW, Bansal E, Garner DC, Smith**
433 **JA, Demicco DD, Schlepner CJ, Aldoghaither RA, Savaliya VA.** 2013. Early
434 clinical observations in prospectively followed patients with fungal meningitis
435 related to contaminated epidural steroid injections. Ann Intern Med **158**:154-161.

- 436 13. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011.
437 MEGA5: molecular evolutionary genetics analysis using maximum likelihood,
438 evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**:2731-
439 2739.
- 440 14. **Lockhart SR, Pham CD, Gade L, Iqbal N, Scheel CM, Cleveland AA, Whitney**
441 **AM, Noble-Wang J, Chiller TM, Park BJ, Litvintseva AP, Brandt ME.** 2013.
442 Preliminary laboratory report of fungal infections associated with contaminated
443 methylprednisolone injections. *J Clin Microbiol* **51**:2654-2661.
- 444 15. **Smith RM, Tipple M, Chaudry MN, Schaefer MK, Park BJ.** 2013. Relapse of
445 fungal meningitis associated with contaminated methylprednisolone. *N Engl J*
446 *Med* **368**:2535-2536.
- 447 16. **Centers for Disease Control and Prevention.** 2012. Multistate fungal
448 meningitis outbreak -- interim guidance for treatment. *MMWR Morb Mortal Wkly*
449 *Rep* **61**:842.
- 450 17. **Zhao Y, Perlin DS.** 2013. Quantitative detection of *Aspergillus* spp. by real-time
451 nucleic acid sequence-based amplification, p. 83-92, *Fungal Diagnostics*.
452 Springer.
- 453 18. **Millon L, Larosa F, Lepiller Q, Legrand F, Rocchi S, Daguindau E, Scherer**
454 **E, Bellanger AP, Leroy J, Grenouillet F.** 2013. Quantitative polymerase chain
455 reaction detection of circulating DNA in serum for early diagnosis of
456 mucormycosis in immunocompromised patients. *Clin Infect Dis* **56**:e95-101.

- 457 19. **Kami M, Fukui T, Ogawa S, Kazuyama Y, Machida U, Tanaka Y, Kanda Y,**
458 **Kashima T, Yamazaki Y, Hamaki T.** 2001. Use of real-time PCR on blood
459 samples for diagnosis of invasive aspergillosis. *Clin Infect Dis* **33**:1504-1512.
- 460 20. **Millon L, Grenouillet F, Crouzet J, Larosa F, Loewert S, Bellanger A-P,**
461 **Deconinck E, Legrand F.** 2010. False-positive *Aspergillus* real-time PCR assay
462 due to a nutritional supplement in a bone marrow transplant recipient with GVH
463 disease. *Med Mycol* **48**:661-664.
- 464 21. **Cuenca-Estrella M, Meije Y, Diaz-Pedroche C, Gomez-Lopez A, Buitrago MJ,**
465 **Bernal-Martinez L, Grande C, San Juan R, Lizasoain M, Rodriguez-Tudela**
466 **JL.** 2009. Value of serial quantification of fungal DNA by a real-time PCR-based
467 technique for early diagnosis of invasive aspergillosis in patients with febrile
468 neutropenia. *J Clin Microbiol* **47**:379-384.
- 469 22. **Pettit AC, Kropski JA, Castilho JL, Schmitz JE, Rauch CA, Mobley BC,**
470 **Wang XJ, Spires SS, Pugh ME.** 2012. The index case for the fungal meningitis
471 outbreak in the United States. *N Engl J Med* **367**:2119-2125.
- 472
- 473
- 474
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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.

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

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 (\pm SD) ^b	Clinical Outcome
Patient 1	F	42	17-Oct-12	Neg	<i>Er</i>	<i>Er</i>	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	<i>Er</i>	38	119	>500	Relapse meningitis,

			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	<i>Er</i>	<i>Er</i>	36	2075	>500	
Patient 4	F	77	9-Oct-12	Neg	<i>Er</i>	<i>Er</i>	30	2550	>500	Relapse soft tissue
			8-Nov-12	Neg	Neg	<i>Er</i>	40	34	>500	phlegmon,
			5-Dec-12	Neg	Neg	<i>Er</i>	38	0	>500	on retreatment, stable
Patient 5	M	72	5-Oct-12	Neg	<i>Er</i>	<i>Er</i>	34	1989	>500	
			19-Dec-12	Neg	Neg	<i>Er</i>	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	<i>Er</i>	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

Patient 7	F	64	11-Oct-12	Neg	<i>Er</i>	<i>Er</i>	30	3996	>500	Completed treatment, asymptomatic, stable
			25-Oct-12	Neg	Neg	Ind	41	43	N/P	
			2-Jan-13	Neg	Neg	Neg	0	7	>500	
			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	<i>Er</i>	<i>Er</i>	39	602	N/P	Completed treatment, asymptomatic, stable
			28-Nov-12	Neg	Neg	Neg	0	85	>500	
			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	<i>Er</i>	<i>Er</i>	<i>Er</i>	31	N/P	N/P	Completed treatment,
			12-Nov-12	Neg	Neg	Ind	44	3106	>500	asymptomatic,

			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	<i>Er</i>	34	2676	>500	
			21-Nov-12	Neg	Neg	Neg	0	66	>500	
			1-Mar-13	Neg	Neg	Neg	0	17	>500	Continues on
			5-Apr-13	<i>En</i>	Neg	Neg	0	157	427 ± 35	treatment (8 months),
			26-Apr-13	Neg	Neg	Neg	0	48	240 ± 14	stable
			28-May-13	Neg	Neg	Neg	0	43	<32	
Patient 11	M	65	4-Oct-12	Neg	<i>Er</i>	<i>Er</i>	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	

Patient 12	F	58	29-Sep-12	Cc	Neg	Neg	0	9080	>500	Completed treatment, asymptomatic, stable
			22-Oct-12	Neg	Neg	Neg	0	173	N/P	
			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	<i>Er</i>	<i>Er</i>	30	1530	>500	Completed treatment, asymptomatic, stable
			9-Nov-12	Neg	Neg	Ind	42	6373	>500	
			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	<i>Er</i>	<i>Er</i>	<i>Er</i>	32	830	>500	Continues on treatment (8 months),stable
			8-Nov-12	Neg	Neg	Neg	0	418	>500	
			18-Dec-12	Neg	Neg	Neg	0	121	>500	
			12-Mar-13	Neg	Neg	Neg	0	50	32 ± 61	

			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	<i>Er</i>	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	<i>Er</i>	<i>Er</i>	34	48	>500	Completed treatment,
			28-Nov-12	<i>En</i>	Neg	<i>Er</i>	38	954	>500	asymptomatic,

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

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