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MAJOR ARTICLE

A Seminested PCR Method for the Diagnosis of Invasive Fungal Infections in Combat Injured

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Background. Among combat injured, invasive fungal infections (IFIs) result in significant morbidity. Cultures and histopathology are the primary diagnostic methods for IFIs, but they have limitations. We previously evaluated a panfungal polymerase chain reaction assay, which was 83% sensitive and 99% specific for angioinvasive IFIs. Here, we evaluated 3 less resource-intensive seminested assays targeting clinically relevant fungi in the order Mucorales and genera *Aspergillus* and *Fusarium*.

Methods. Formalin-fixed paraffin-embedded tissue specimens from a multicenter trauma IFI cohort (2009-2014) were used. Cases were US military personnel injured in Afghanistan with histopathologic IFI evidence. Controls were patients with similar injury patterns and no laboratory IFI evidence (negative culture and histopathology). Seminested assays specific to Mucorales (V4/V5 regions of 18S rDNA), *Aspergillus* (mitochondrial tRNA), and *Fusarium* (internal transcribed spacer [ITS]/28A regions of DNA) were compared with a panfungal assay amplifying the internal transcribed spacer 2 region of rDNA and to histopathology.

Results. Specimens from 92 injury sites (62 subjects) were compared with control specimens from 117 injuries (101 subjects). We observed substantial agreement between the seminested and panfungal assays overall, especially for the order Mucorales. Moderate agreement was observed at the genus level for *Aspergillus* and *Fusarium*. When compared with histopathology, sensitivity and specificity of seminested assays were 67.4% and 96.6%, respectively (sensitivity increased to 91.7% when restricted to sites with angioinvasion).

Conclusions. Prior studies of seminested molecular diagnostics have focused on culture-negative samples from immunocompromised patients. Our findings underscore the utility of the seminested approach in diagnosing soft-tissue IFIs using formalin-fixed paraffin-embedded tissue samples, especially with angioinvasion.

Keywords. combat; invasive fungal wound infection; mucormycosis; PCR-based assays; trauma.

During the war in Afghanistan, ~5% of battlefield trauma admissions were complicated by invasive fungal wound infections (IFIs) [1]. Crude mortality was 6%, and 34% of IFIs required changes in amputation level, with 15% requiring high-level amputations (ie, occurring at hip level) [2]. In civilian settings, ~59% of primary cutaneous mucormycosis are complications of trauma [3]. Because of diagnostic limitations, IFIs present unique clinical challenges [3, 4]. Mortality and morbidity associated with combat-related IFIs vary based on infection severity (ie, angioinvasion) and infecting organisms, with infections resulting from mucormycetes more severe [5, 6], underscoring the need to rapidly identify IFIs and their etiology.

Clinical outcomes in military and civilian series are adversely impacted by delays in microbiologic diagnosis and, thereby, definitive treatment [6–8]. Conventionally, histopathology and culture are gold standards of IFI diagnosis [9]; however, histopathology cannot reliably provide species-level identification and cultures are insensitive, often delayed, and, unlike bacterial cultures, require significant expertise to provide species-level identification [10]. Molecular methods promise more expedient and accurate diagnostic results [9, 11–14]. Using formalin-fixed paraffin-embedded (FFPE) tissue samples, we previously explored the utility of a panfungal assay (ie, assay to identify any fungus) for supporting IFI diagnosis from combat casualties [15]. When compared with histopathology, this technique was highly specific (99%), but demonstrated variable sensitivity depending on

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occurrence of angioinvasion (63% overall, 83% with specimens from angioinvasive sites). The results also confirmed culture observations that fungi of the order Mucorales and genera *Aspergillus* and *Fusarium* dominate in this setting [5, 6].

Panfungal techniques require genetic sequencing of all outputs to evaluate whether results are clinically meaningful, whereas seminested polymerase chain reaction (PCR) assays (ie, targeted to specific fungus) using directed primers have the potential to provide preliminary information about the presence or absence of clinically relevant fungi without sequencing (and is therefore faster), albeit sequencing is still required for species-level identification. We previously examined the performance of seminested assays using a subset of samples evaluated by the panfungal assay and observed slightly higher sensitivity, suggesting further evaluation with seminested assays was warranted [15]. Our objective for the present study was to assess the diagnostic performance of seminested assays against the gold standards of histopathology and culture and to assess concordance with the panfungal assay.

METHODS

Study Design

The FFPE tissue specimens from wounded military personnel were collected for clinical care and were approved for use by the institutional review board of the Uniformed Services University (Bethesda, MD). Histopathology and culture results used for this retrospective analysis were collected through the Trauma Infectious Disease Outcomes Study, an observational, multicenter study focused on examining outcomes of battlefield-related infections [16]. Information on demographics, injury characteristics, and trauma care were obtained from the Department of Defense Trauma Registry [17].

Study Population

As previously described, patients were eligible for inclusion if they sustained a trauma-related open injury in Afghanistan (6/1/2009-12/31/2014), were admitted to participating US military hospitals (Walter Reed National Military Medical Center or Brooke Army Medical Center) after being evacuated through Landstuhl Regional Medical Center, Germany, and had surgical specimens available [15]. The IFI definitions developed by the 2008 Mycoses Study Group were modified for use in trauma patients [5, 18]. To qualify as an IFI, we required both persistent necrosis and laboratory evidence of fungus (ie, presence of filamentous fungus on culture or histopathology following ≥2 surgical debridements) [1, 5]. We further categorized IFIs based on histopathology as Proven (presence of angioinvasion), Probable (presence of fungal hyphae, but no angioinvasion), and Possible (positive cultures only). For this analysis, case patients had either Proven or Probable IFIs. Among 70 patients with Proven/Probable IFIs, 62 had tissue specimens available and were included. A hospital-based clinical practice guideline involving sampling of patients at risk for IFIs was implemented in 2011 [19]. Control patients were identified from this population if they had specimens submitted to assess for IFIs based on a clinical suspicion and were negative for fungus on histopathology and culture.

Tissue Specimens

Using standard operating procedures, Clinical Laboratory Improvement Amendments/College of American Pathologists–certified hospital laboratories processed surgical specimens obtained in the operating room. To reduce potential for contamination during sectioning, tissue blocks were surface decontaminated using an ethanol rinse, sterile distilled water rinse, and DNAZap (ThermoFisher Scientific, Waltham, MA) and sectioned into 5- to 20-µm-thick sections using sterile microtomes, discarding the first 5-µm section of the block. Samples were shipped to the Advanced Nucleic Acids Core Facility at University of Texas Health Science Center San Antonio (UTHSCSA) for fungal DNA extraction, PCR amplification, and sequencing [15]. As part of original panfungal analysis, 20% of blinded specimens were shipped to LADR GmbH, Medizinisches Versorgungszentrum Dr. Kramer & Kollegen (Germany), for analysis with seminested assays [15].

PCR-Based Assays

As previously described [15, 20], the panfungal assay developed by the Advanced Nucleic Acids Core Facility at UTHSCSA amplified the internal transcribed spacer 2 region of fungal rDNA, a constitutively expressed and conserved locus, using primers for flanking regions ITS3 (5′-GCATCGATGAAG AACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).

The seminested assays were specific to the order Mucorales and genera *Aspergillus* and *Fusarium* because they were most frequently identified from wound cultures (Supplementary Table 1). Seminested techniques used by LADR for the original subset analysis [15] were used by the UTHSCSA team for this analysis. Fungal organisms of the order Mucorales were identified by amplifying the V4 and V5 regions of the 18S rDNA using the outer primers ZM1 (5′-ATTACCATGAGCAAATCA GA-3′) and ZM2 (5′-TCCGTCAATTCCTTTAAGTTTC-3′) and then primers ZM1 and ZM3 (5′-CAATCCAAGAATTTC ACCTCTAG-3′), resulting in seminested products [21]. *Aspergillus* spp. were identified by amplifying *Aspergillus fumigatus* mitochondrial transfer RNA by using primers P2 (5′-CT TTGGTTGCGGGTTTAGGGATT-3′) and Asp2 (5′-GGGAG TTCAAATCTCCCTTGGG-3′) followed by seminested PCR using primers P2 and P1 (5′-GAAAGGTCAGGTGTTCGA GTCAC-3′) [21, 22]. *Fusarium* spp. were identified by amplifying a DNA sequence within the ITS/28s region of *Fusarium* spp. using outer primers FUS I (5′-AGTATTCTGGCGGGCATG CCTGT-3′) and FUS II (5′-ACAAATTACAACTCGGGCCC GAGA-3′) [23], and a FUS III primer (5′-CCGTTAC

TGAGGCAATCCCTGTT-3′) was constructed to increase the sensitivity of the assay, resulting in a seminested product.

All 3 seminested assays used the same protocol. Reaction mixtures of the primary PCRs consisted of 10 μL of previously extracted DNA in a total volume of 50 μL, with final concentrations of 10 mM Tris/HCl (pH 8.3), 50 mM KCl, and 2.5 mM MgCl2 (10× Perkin-Elmer buffer II plus MgCl2 solution; Roche Molecular Systems, Branchburg, NJ); 1 μM of each primer of the outer primer sets (ZM1/ZM2, P2/Asp2, and FUS I/FUS II, respectively; Roth, Karlsruhe, Germany); 1.5 U of AmpliTaq DNA polymerase (Roche); and 100 μM of each dNTP (Promega, Madison, WI). Identical reaction mixtures were used for the nested PCRs except that 1 μL of the first reaction, 50μM of each dNTP, and 1 μM of each inner primer (ZM1/ZM3, P2/P1, FUSI/FUSIII) were used.

All reaction mixtures were thermally cycled once at 94 °C for 5 minutes, 35 times at 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1 minute, and then once at 72 °C for 5 minutes. To validate the presence of amplifiable DNA and absence of inhibitory substances, a PCR was performed using the primer set G1 (5′-GAAGAGCCAAGGACAGGTAC-3′) and G2 (5′-CAACTT CATCCACGTTCACC-3′) targeting the human β-globin gene (nucleotides 70400-70667; GenBank accession number NG_ 000007.3; https://www.ncbi.nlm.nih.gov/nuccore/NG_000007.3). The conditions were as described previously except that 5 μL of DNA extract and a final concentration of 3 mM $MgCl_2$ were used, and the extension time was reduced from 1 minute to 45 seconds. When the result was negative, DNA extraction was repeated if enough material was available. All PCRs were run in an Applied Biosystems MiniAmp Thermal Cycler (ThermoFisher Scientific). PCR products were analyzed by electrophoresis in ethidium bromide stained 1.8% agarose gels. Amplicons of the primary PCRs using DNA extracted from laboratory strains of *A fumigatus* and *Absidia corymbifera* were cloned as described in detail previously [24]. Cloned plasmid DNA (10 μL containing 100 fg [~5000 genome equivalents]) was used as a positive control in every PCR assay. Sterile water was included in the DNA extraction after every fifth sample and used in the PCR assays, and reaction mixtures without DNA were run in the first and nested PCRs to monitor contamination.

Nested PCR products were sequenced by a semiautomated system (Applied Biosystems Division, Perkin-Elmer Biosystems, Foster City, CA) and sequences were used for a BLAST search of the GenBank database [24]. A PCR assay was considered positive if the product obtained from the *Aspergillus* or *Fusarium* PCR was identical to *Aspergillus* or *Fusarium* sequences in GenBank, respectively, or if the product amplified by Mucorales PCR showed 100% homology to an 18S rDNA sequence of a member of the order Mucorales.

Statistics

Patient characteristics were compared using Fisher exact test or chi-square test (as appropriate) for categorical variables and Wilcoxon rank-sum test for continuous variables. $P < .05$ was considered statistically significant.

Because patients could have multiple wounds with multiple debridements, each patient could contribute >1 specimen from the same or different injury site. All analyses were performed at the injury site level. If multiple specimens were obtained from injury sites, findings were pooled and analyzed.

Performance characteristics of seminested assays were compared with histopathology. Because of the lack of specimens, 4 injury sites (3 cases, 1 control) included in the panfungal analysis were not included in the seminested analysis. In the original panfungal analysis [15], identification of any fungal pathogen, excluding skin or environmental contaminants, was considered positive. For an accurate comparison with the seminested assays, the panfungal findings were only considered positive in this analysis if they identified pathogens targeted by the seminested assays (ie, order Mucorales or genera *Aspergillus* or *Fusarium*).

Measures of concordance/agreement between the panfungal and seminested methods and conventional cultures were calculated using the Cohen kappa coefficient with 95% confidence interval; standard thresholds of kappa = 0.800 representing substantial to almost perfect agreement, kappa = 0.400 to 0.799 representing moderate agreement, and kappa ≤ 0.399 representing poor agreement [25]. Results were considered concordant if fungal identifications matched at the site level. Because the seminested assay cannot differentiate between *Apophysomyces* and *Saksenaea*, for measures of concordance between the panfungal and seminested assays, specimens identified as *Apophysomyces elegans* were included with *Saksenaea* spp. because the target sequences of the LADR Mucorales assay were identical. These, along with diagnostic performance parameters, were calculated using SAS Version 9.4 (SAS, Cary, NC).

RESULTS

Study Population

A total of 163 patients (62 IFI case patients contributing 161 specimens from 92 injury sites and 101 control patients contributing 127 specimens from 117 injury sites) were examined (Figure 1). Patients were young (median age, 24 years), male (100%), and primarily injured via a blast mechanism (86.5%), with the case patients having greater blood transfusion requirements (median of 33.5 vs 10 units; $P < .001$) and more lower extremity amputations (76% vs 26%; $P < .001$) compared with control patients (Supplementary Table 2).

Comparison of Panfungal and Seminested Assays

Among the 209 case and control injury sites, seminested assays identified fungal DNA in 66 sites (31.6%), which included 4 false positives, whereas the panfungal assay identified fungal

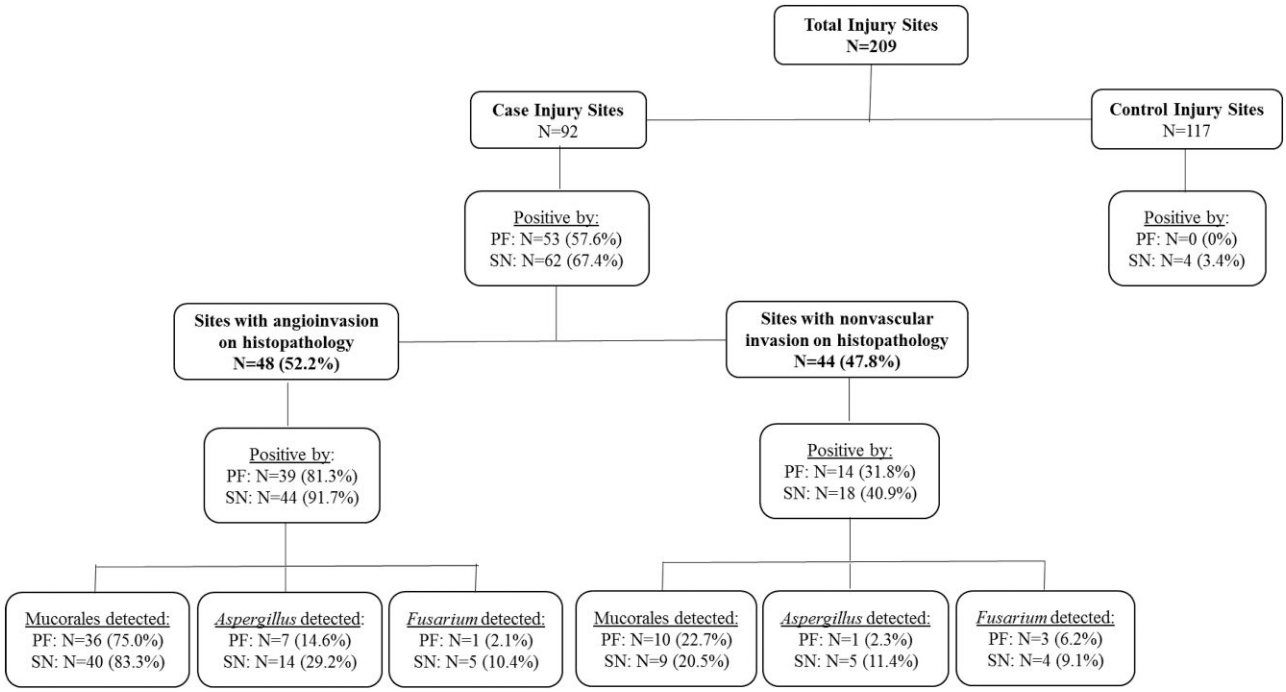

Figure 1. Injury site level results from the seminested (SN) and panfungal (PF) assays. Case injury sites are those with fungal nonvascular tissue invasion or angioinvasion from 62 patients. Control injury sites are those that are negative for fungal histopathology and fungal cultures from 101 patients. Positive is defined as identification of fungal DNA by polymerase chain reaction.

Table 1. Comparison of Seminested and Panfungal Assays

Fungal Positivity Findings, No. (%)	Seminested Assay Results	Panfungal Assay Results	Kappa Coefficient, Median (IQR)
Specimens assessed using both techniques			
No fungal DNA	143 (68.4)	156 (74.6)	0.801 (0.712, 0.891)
Any fungal DNA	66[a] (31.6)	53 (25.4)	
Fungi identified at genus level			
Order Mucorales	51 (24.4)	46 (22.0)	0.879 (0.802, 0.956)
Aspergillus spp.	20 (9.6)	8 (3.8)	0.471 (0.242, 0.701)
Fusarium spp.	10 (4.8)	4 (1.9)	0.559 (0.248, 0.871)

Abbreviation: IQR, interquartile range.

[a]Includes 4 control injury sites with positive findings with the seminested assays.

DNA in 53 sites (25.4%; Table 1; Supplementary Table 3). There was substantial agreement between the 2 techniques (kappa: 0.801; Table 1). When stratified at order/genera level, there was substantial agreement for the order Mucorales (kappa: 0.879), but moderate agreement for genera *Aspergillus* and *Fusarium* (kappa: 0.471 and 0.559, respectively).

Among 48 injury sites with documented angioinvasion (Proven IFI), seminested assays identified fungal DNA in 44 sites and panfungal in 39 sites (91.7% vs 81.3%; Table 2). Among injury sites with nonvascular tissue invasion (Probable IFI), 18 (40.9%) and 14 (31.8%) sites were positive for fungal DNA using the seminested and panfungal assays, respectively (Figure 1, Table 2).

Using seminested assays, case sites primarily showed evidence of fungi of the order Mucorales (49/92, 53.3%), whereas fungi of the genera *Aspergillus* spp. and *Fusarium* spp. were found in 20.7% and 9.8%, respectively (Table 2). Among sites with angioinvasion, fungi of the order Mucorales dominated (either alone or in combination with fungi of the genera *Aspergillus* and *Fusarium*) at 83.3%.

Comparison of Seminested Assays and Conventional Fungal Culture

Culture results were available for 84 (91% of 92) injury sites (Table 3; Supplementary Tables 4–7). Fungal cultures were positive in 63.1% of injury sites, whereas seminested assays identified fungal DNA in 69.1%. There was significant discordance between these results at specific injury sites reflected in a kappa coefficient of 0.180, suggesting poor agreement (Table 3; Supplementary Table 4). Among specific fungal types (Table 3; Supplementary Tables 5–7), there was poor agreement for fungi of the order Mucorales (kappa: 0.326) and genera *Aspergillus* and *Fusarium* (kappa: 0.300 and 0.230, respectively). Among 74 injury sites with collection of cultures and histopathology specimens within 24 hours of each other, fungus was identified in cultures from 39 injury sites (52.7%) compared with 52 injury sites (70.3%) with seminested assays (Table 3). Discordance was observed between the results at

Table 2. Frequency of Identification of Fungal DNA With PCR-based Assays Stratified by Histopathological Identification

PCR Findings, No. (%)[a]	Seminested Assay Results		Panfungal Assay Results	
	Sites With Angioinvasion (N = 48)	Sites With Nonvascular Tissue Invasion (N = 44)	Sites With Angioinvasion (N = 48)	Sites With Nonvascular tissue Invasion (N = 44)
Fungal DNA detected	44 (91.7)	18 (40.9)	39 (81.3)	14 (31.8)
Order Mucorales	40 (83.3)	9 (20.5)	36 (75.0)	10 (22.7)
Aspergillus spp.	14 (29.2)	5 (11.4)	7 (14.6)	1 (2.3)
Fusarium spp.	5 (10.4)	4 (9.1)	1 (2.1)	3 (6.2)
Combinations				
Mucorales only	26 (54.2)	9 (20.5)	32 (66.7)	10 (22.7)
Aspergillus only	2 (4.2)	5 (11.4)	2 (4.2)	1 (2.3)
Fusarium only	2 (4.2)	4 (9.1)	0	3 (6.2)
Mucorales plus *Aspergillus* spp.	11 (22.9)	0	4 (8.3)	0
Aspergillus plus *Fusarium* spp.	0	0	1 (2.1)	0
Mucorales plus *Fusarium* spp.	2 (4.2)	0	0	0
Mucorales plus *Aspergillus* spp. plus *Fusarium* spp.	1 (2.1)	0	0	0

Abbreviation: PCR, polymerase chain reaction.

[a]Genus-level categories are not mutually exclusive.

specific sites with a kappa coefficient of 0.143. Agreement was moderate (kappa: 0.405) when stratified for organisms of the order Mucorales, but poor agreement was observed among *Aspergillus* spp. and *Fusarium* spp. (kappa: 0.184 and 0.136, respectively).

Forty-four of the 48 injury sites with evidence of angioinvasion on histopathology (91.7%) had culture results available (Table 3). Of these, fungal DNA was identified in 41 sites (93.2%) by seminested assays compared with 32 (72.7%) by culture; however, because of discordance of specific samples, the kappa coefficient suggested no agreement (kappa: −0.122; Table 3, Supplementary Table 4). We observed moderate agreement between seminested assays and culture for *Aspergillus* spp. (kappa: 0.413) and poor agreement for *Fusarium* spp. (kappa: 0.259) and for the order Mucorales (kappa: 0.101; Table 3, Supplementary Tables 5–7). Forty sites were cultured within 24 hours of histopathology specimen collection. Of these, seminested assays identified fungal DNA in 37 sites (92.5%) compared with 27 sites (67.5%) by culture; however, discordance was high with a kappa coefficient of 0.004. Concordance was low, with the best agreement seen for organisms of the order Mucorales, albeit poor (kappa: 0.225).

Performance Characteristics of the Seminested Assays

Overall sensitivity and specificity of the seminested assay was 67.4% and 96.6%, respectively (Table 4). Among sites with documented angioinvasion, sensitivity of the seminested assay improved to 91.7%. The overall negative predictive value of the seminested assays was 79.0% and improved to 96.6% specimens in specimens with angioinvasion. In comparison, the panfungal assay was 57.6% sensitive (improved to 81.3% with documented angioinvasion) and 100% specific with a negative predictive value of 75.0% overall and 92.9% with angioinvasion.

DISCUSSION

We previously described the performance characteristics of a panfungal assay for the diagnosis of trauma-related IFIs [15], and here, we extend those observations by evaluating 3 seminested assays designed to detect fungi of the order Mucorales and genera *Aspergillus* and *Fusarium*. Selection of fungal targets for the seminested assays were predicated on a review of published literature, conventional culture results, and our prior panfungal work [3, 5–7, 15, 26, 27]. As observed in the civilian setting, using seminested assays, fungi of the order Mucorales dominated; however, fungi of the genera *Aspergillus* and *Fusarium* were also isolated from combat injuries sustained in Afghanistan [3, 5, 28]. The occurrence of infections with *Aspergillus* and *Fusarium* may have resulted from combat casualties experiencing immunosuppression from massive blood transfusions (>20 units with 24 hours postinjury), as well as trauma-associated immune paralysis [29, 30].

Despite theoretical concerns that DNA degradation from formalin fixation would limit ability to use samples for fungal identification [31], our results suggest it was feasible to perform seminested assays on FFPE samples. Using FFPE for diagnostic purposes is an advantage in areas of the world where laboratory services are rudimentary with limited capacity to identify IFIs. With seminested assays using shorter target sequences compared with the panfungal approach, we observed slightly better overall sensitivity (67.4% vs 57.6%) and negative predictive values (79.0% vs 75.0%); however, neither assay is highly sensitive. The specificity of both assays was excellent (100% for panfungal and 96.6% for seminested). Both assays demonstrated greater sensitivity in FFPE tissue samples exhibiting angioinvasion, perhaps reflective of the higher fungal burden in specimens with documented angioinvasion [32]. Even among sites with

Table 3. Frequency of Identification of Fungi by Culture vs Seminested Assays Stratified by Extent of Angioinvasion on Histopathology

Growth of Filamentous Fungi, No. (%)	Any Type of Positive Histopathology			Histopathology Positive for Angioinvasion		
	Culture Results	PCR Results	Kappa Coefficient, Median (IQR)	Culture Results	PCR Results	Kappa Coefficient, Median (IQR)
Cultures collected, No. of injury sites	84	84		44	44	
No fungi	31 (36.9)	26 (31.0)	0.180 (–0.035, 0.395)	12 (27.3)	3 (6.8)	–0.122 (–0.241, –0.004)
Any fungi	53 (63.1)	58 (69.1)		32 (72.7)	41 (93.2)	
Order Mucorales	31 (36.9)	46 (54.8)[a]	0.326 (0.139, 0.514)	20 (45.5)	37 (84.1)[a]	0.101 (–0.096, 0.298)
Aspergillus spp.	21 (25.0)	18 (21.4)[b]	0.300 (0.067, 0.533)	13 (29.6)	14 (31.8)[b]	0.413 (0.123, 0.702)
Fusarium spp.	15 (17.9)	9 (10.7)	0.230 (–0.032, 0.493)	11 (25.0)	5 (11.4)	0.259 (–0.060, 0.579)
Combinations						
Mucorales only	20 (23.8)	32 (38.1)		11 (25.0)	23 (52.3)	
Aspergillus only	14 (16.7)	6 (7.1)		7 (15.9)	2 (4.6)	
Fusarium only	7 (8.3)	6 (7.1)		4 (9.1)	2 (4.6)	
Mucorales plus *Aspergillus* spp.	4 (4.8)	11 (13.1)		3 (6.8)	11 (25.0)	
Aspergillus plus *Fusarium* spp.	1 (1.2)	0		1 (2.3)	0 (0)	
Mucorales plus *Fusarium* spp.	5 (6.0)	2 (2.4)		4 (9.1)	2 (4.6)	
Mucorales plus *Aspergillus* spp. plus *Fusarium* spp.	2 (2.4)	1 (1.2)		2 (4.6)	1 (2.3)	
Cultures collected within 24 h of histopathology specimen collection, No. of injury sites	74	74		40	40	
No fungi	35 (47.3)	22 (29.7)	0.143 (–0.068, 0.355)	13 (32.5)	3 (7.5)	0.004 (–0.215, 0.223)
Any fungi	39 (52.7)	52 (70.3)		27 (67.5)	37 (92.5)	
Order Mucorales	25 (33.8)	42 (56.8)	0.405 (0.222, 0.587)	19 (47.5)	33 (82.5)	0.225 (0.008, 0.443)
Aspergillus spp.	11 (14.9)	14 (18.9)	0.184 (–0.082, 0.451)	5 (12.5)	12 (30.0)	0.214 (–0.091, 0.520)
Fusarium spp.	8 (10.8)	9 (12.2)	0.136 (–0.153, 0.426)	7 (17.5)	5 (12.5)	0.220 (–0.160, 0.599)
Combinations						
Mucorales only	20 (27.0)	30 (57.7)		15 (37.5)	21 (52.5)	
Aspergillus only	10 (13.5)	4 (5.4)		5 (12.5)	2 (5.0)	
Fusarium only	4 (5.4)	6 (8.1)		3 (7.5)	2 (5.0)	
Mucorales plus *Aspergillus* spp.	1 (1.4)	9 (12.2)		0	9 (22.5)	
Aspergillus plus *Fusarium* spp.	0	0		0	0	
Mucorales plus *Fusarium* spp.	4 (5.4)	2 (2.7)		4 (10.0)	2 (5.0)	
Mucorales plus *Aspergillus* spp. plus *Fusarium* spp.	0	1 (1.4)		0	1 (2.5)	

Abbreviations: IQR, interquartile range; PCR, polymerase chain reaction.

[a] *Saksenaea* spp./*Apophysomyces elegans* (N = 26 for sites with angioinvasion; N = 8 for sites with nonvascular tissue invasion); *Mucor circinelloides* (N = 3 for sites with angioinvasion; N = 1 for sites with nonvascular tissue invasion).

[b] *Aspergillus terreus* (N = 1 for sites with angioinvasion); *A flavus* (N = 9 for sites with angioinvasion; N = 3 for sites with nonvascular tissue invasion); *A fumigatus* (N = 6 for sites with angioinvasion; N = 1 for sites with nonvascular tissue invasion).

angioinvasion, seminested assays had a higher sensitivity than the panfungal approach (91.7% vs 81.3%), which is not surprising because the panfungal target (internal transcribed spacer 2 region) is roughly twice the length of nested PCR targets. Additionally, the seminested assays use 2 amplifications, the first being the FFPE template DNA and the second being any amplicon produced from the FFPE template. Although agreement between the seminested and panfungal assay was high among the mucormycetes, it was moderate for specimens with fungi of the genera *Aspergillus* and *Fusarium*. We hypothesize that the observed lower agreement may be due to the general insensitivity of the ribosomal locus for identifying *Aspergillus* and *Fusarium* spp. Without inclusion of a second nonribosomal locus sequence (eg, translation elongation factor EF-1α), multiple species may have 100% identity using ribosomal sequences as targets. In contrast, greater diversity of the order Mucorales allows fungal identification with a single sequence, depending on the species. Moreover, order Mucorales fungi are aseptate with nuclei concentrated in metabolically active regions [33]. Thus, if fungal DNA is detected, the amount is generally high because of a greater number of fungal nuclei. Because *Aspergillus* and *Fusarium* spp. form septa with only 1 nucleus between them, there is potentially less amplifiable DNA per section.

Our findings suggest that the seminested assays may have greater clinical utility compared with the panfungal approach, as the latter relies on sequencing all products to determine clinical relevance of results, lengthening the diagnostic process. For example, in our prior work with the panfungal assay (data not shown), *Malasezzia,* a common skin contaminant, was often identified (recovered from 30.5% of sites with positive fungal histopathology) [15]. In contrast, seminested assays target specific organisms and offer evidence of the presence (or absence) of a specific order (eg, Mucorales) or genus (eg, *Aspergillus*,

Table 4. Seminested PCR-based Assay Test Performance Compared With Histopathology

Characteristic, % (95% CI)	Specimens From Sites With Any Type of Histopathology (N = 92)	Specimens From Sites With Angioinvasion (N = 48)
Sensitivity	67.4 (56.8-76.8)	91.7 (80.0-97.7)
Specificity	96.6 (91.5-99.1)	96.6 (91.5-99.1)
Positive predictive value	93.9 (85.4-97.6)	91.7 (80.7-96.7)
Negative predictive value	79.0 (73.7-83.5)	96.6 (91.7-98.6)
False-positive rate	3.4 (0.9-8.5)	3.4 (0.9-8.5)
False-negative rate	32.6 (23.2-43.2)	8.3 (2.3-20.0)

Abbreviations: CI, confidence interval; PCR, polymerase chain reaction.

Fusarium). Given the high specificity of the seminested assays, in the appropriate clinical setting, initial positive results (even without sequencing) may be considered clinically relevant and used to guide targeted antifungal therapy. Seminested assays may also be adaptable to quantitative PCR depending on whether conserved regions of the amplicon could be identified for probe design.

Although we noted discordance between the cultures and seminested assay results, the discrepancies were less pronounced when cultures and histopathologic specimens were contemporaneous (±24 hours). Discordance between culture and seminested assays for the fungi of the order Mucorales are not unexpected given the recognized reduced sensitivity of cultures in identifying mucormycosis [34, 35] and, as described previously, the lower sensitivity of the molecular assays for the genera *Aspergillus* spp. and *Fusarium* spp. Our results suggest that culture and the seminested assays are complementary. To maximize yield and compensate for the time-delayed nature of culture results, particularly for fungi of the genera *Aspergillus* and *Fusarium*, all 3 testing strategies should be pursued (ie, histopathology, culture, and molecular methods) in accordance with current guidelines [36].

Although further optimization of the seminested assays is needed, our study demonstrates that, where the capability exists, seminested assays could offer early additive diagnostic information of clinical relevance and are especially sensitive at identifying fungi in specimens with angioinvasion. This study demonstrates the order Mucorales as the predominant fungi, either alone or in combination with fungi of the genera *Aspergillus* and *Fusarium*, in trauma-related IFIs, particularly among those with angioinvasion, reflecting trends from South Asia [37, 38]. Taken together, in the right geographic setting and irrespective of culture results, our findings support use of empiric antifungal coverage (plus surgical debridements) for the order Mucorales for angioinvasive trauma-related IFIs, per current military guidelines [36].

Diagnostics performance evaluation is limited by the accuracy of the reference test against which the diagnostic is measured. Thus, our study is limited by the accuracy of histopathology in detecting and classifying IFIs in our population. Histopathological examination with use of special stains (eg, Gomori methenamine silver, periodic acid-Schiff) plus routine hematoxylin and eosin staining was associated with a 15% false-negative rate [39]. The PCR test itself is limited by degradation of DNA from age and sample processing techniques, and like conventional methods, is affected by delays in tissue sampling, processing, and use of antifungals, the extent of which is uncertain [40]. Further, there is the potential of contamination; however, the timing of specimen collection and techniques used here limited contamination risk. Also, while pooling samples from multiple debridements likely improved diagnostic yield, there was the potential of bias, so performance on single specimens will need to be assessed. Our cohort was large but homogeneous, comprised primarily of IFIs in blast-injured patients; however, we believe the results can be generalized to other trauma/natural disaster related settings.

Overall, our study suggests the potential clinical utility of seminested assays to provide clinically meaningful information (ie, presence or absence of fungi of the order Mucorales and genera *Aspergillus* and *Fusarium*) within a reasonable time frame to support diagnosis of angioinvasive trauma-related IFIs and guide antifungal use. Prospective studies that evaluate fresh tissue samples contemporaneous to injury are needed to further evaluate the clinical utility of this promising diagnostic technique.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Patient consent statement. Data and specimens were collected through an institutional review board–approved waiver of documentation of informed consent for use of deidentified data not obtained through interaction or intervention with human subjects.

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Potential conflicts of interest. The authors have no conflicts to declare.

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