

REPORT DOCUMENTATION PAGE					Form Approved OMB No. 0704-0188	
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to the Department of Defense, Executive Service Directorate (0704-0188). Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ORGANIZATION.						
1. REPORT DATE (DD-MM-YYYY) 12-09-2018		2. REPORT TYPE Technical			3. DATES COVERED (From - To) June 2009 - December 2014	
4. TITLE AND SUBTITLE Tissue-Based Molecular Diagnostics Evaluation in Combat-Related Invasive Fungal Wound Infections				5a. CONTRACT NUMBER		
				5b. GRANT NUMBER D_MIDCTA_I_12_J2_296		
				5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Anuradha Ganesan, MD David Tribble, MD, DrPH				5d. PROJECT NUMBER		
				5e. TASK NUMBER		
				5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Infectious Disease Clinical Research Program, Department of Preventive Medicine and Biostatistics, Uniformed University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20184					8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Uniformed University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20184					10. SPONSOR/MONITOR'S ACRONYM(S) USU	
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT approved for public release, distribution unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT Trauma-related invasive fungal wound infections (IFIs) are associated with significant morbidity and mortality and early identification and treatment are critical. We assessed a panfungal PCR-based sequencing assay for rapid identification of filamentous fungi in formalin-fixed paraffin-embedded (FFPE) specimens obtained from combat casualties injured in Afghanistan. The internal transcribed spacer 2 (ITS2) region of the fungal ribosomal repeat was amplified and sequenced. The PCR results were compared with findings from histopathology and/or culture. We included 64 case subjects (contributing 95 sites) and 102 controls (contributing 118 sites). Compared to histopathology, panfungal PCR was specific (99%), but not as sensitive (63%); however, sensitivity improved to 83% in specimens from sites with angioinvasion. Panfungal PCR identified fungi of the order Mucorales in 33 of 44 sites with angioinvasion (75%), whereas fungal culture was positive in 20 of 44 sites (45%). Saksena spp. were the dominant fungi identified by PCR in specimens from angioinvasion sites (57%). Panfungal PCR is specific, albeit with lower sensitivity, and performs better at identifying fungi of the order Mucorales than culture.						
15. SUBJECT TERMS invasive fungal wound infection; mucormycosis; PCR-based assay; combat-related trauma; blast trauma						
16. SECURITY CLASSIFICATION OF: a. REPORT unclassified			17. LIMITATION OF ABSTRACT SAR		18. NUMBER OF PAGES 74	
b. ABSTRACT unclassified					19a. NAME OF RESPONSIBLE PERSON David Tribble	
c. THIS PAGE unclassified					19b. TELEPHONE NUMBER (Include area code) 240-694-2984	

TECHNICAL REPORT

Tissue-Based Molecular Diagnostics Evaluation in Combat-Related Invasive Fungal Wound Infections

Meeting held on November 1, 2017, Bethesda, MD



Report generated by the Infectious Disease Clinical Research Program,
Trauma Infectious Disease Outcomes Study (TIDOS)

Defense Medical Research and Development Program [DMRDP Grant #
D_MIDCTA_I_12_J2_296]

Anuradha Ganesan, MD (Principal Investigator); Phone: (301) 295-2972;
anuradha.ganesan.ctr@mail.mil;

Infectious Disease Clinical Research Program, Department of Preventive Medicine and
Biostatistics, Uniformed Services University of the Health Sciences; Walter Reed National
Military Medical Center; Henry M. Jackson Foundation for the Advancement of Military
Medicine, Inc.

David Tribble, MD, DrPH (TIDOS Director); Phone (301) 816-8404; david.tribble@usuhs.edu

TIDOS Director, Infectious Disease Clinical Research Program, Department of Preventive
Medicine and Biostatistics, Uniformed Services University of the Health Sciences;

EXECUTIVE SUMMARY

Purpose: To develop and assess the performance characteristics of a PCR-based molecular assay for the rapid identification of filamentous fungi in formalin-fixed paraffin-embedded (FFPE) tissue specimens obtained during surgical debridement from combat casualties injured in Afghanistan, as well as evaluating the concordance of histopathological and microbiological results with those obtained by PCR-based methods.

Investigative Methods: Using data from the Trauma Infectious Disease Outcomes Study (TIDOS), we identified and characterized all subjects with laboratory evidence of a fungal infection. Using a standardized operating procedure, specimens from patients with positive histopathology (fungal evidence of angioinvasion or nonvascular tissue invasion) were procured by the Pathology service at the Walter Reed National Military Medical Center, Brooke Army Medical Center, and Landstuhl Regional Medical Center. Specimens submitted for histopathologic examination from subjects with similar patterns of injury, but were negative for fungal elements served as negative controls. Blinded specimens (positive histopathology and negative controls) were submitted for evaluation with a panfungal PCR at the University of Texas Health Sciences Center San Antonio, Texas, as well as with a semi-nested PCR at a commercial laboratory (LADR GmbH MVZ Dr. Kramer & Kollegen) in Germany. Fungal DNA was extracted from formalin-fixed tissue samples and paraffin impregnated tissue blocks utilizing sterile techniques, PCR-amplified, and then sequenced. For fungal identification, the ITS2 region in the fungal ribosomal DNA locus were amplified using previously described methods. Results obtained from the PCR were compared with results obtained on histopathology/and or culture.

Main Findings:

PCR Diagnostic Platform

- a) Overall, in comparison to the gold standard of histopathology, the panfungal PCR-based assay was very specific (99%) but not as sensitive (63%). The sensitivity of the panfungal PCR improved to 83% in specimens with documented angioinvasion.
- b) The panfungal PCR performed better than traditional cultures at identifying fungi of the order Mucorales, when compared with traditional culture based methods. Using the panfungal PCR, fungi of the order Mucorales were identified in 77% of sites with positive histopathology (37 of 48 sites with fungal DNA identified), while cultures yielded fungi of the order Mucorales in 45% of the histopathology positive sites (25 of 55 sites with fungal growth in cultures collected within 24 hours of histopathology specimen)
- c) *Saksenaea* spp., (belonging to the order Mucorales) were the dominant fungi identified by the panfungal PCR in approximately half of all sites with positive histopathology
- d) A semi-nested PCR directed against fungi of the genus *Aspergillus* performed better at identifying these organisms in comparison to the panfungal PCR assay

Risk Assessment and Clinical Management

Subjects with laboratory evidence a fungal infection were examined with the aim of classifying them into risk groups that could be used for clinical management, along with assessment of how previously proposed definitions performed at predicting risk for an IFI.

- a) The lack of ongoing necrosis and the absence of fungi of the order Mucorales, genus *Aspergillus* and *Fusarium* identifies a group of individuals in whom antifungals can be withdrawn with close clinical follow-up
- b) Approximately 62% of subjects with an IFI or High Suspicion of IFI met the definition of a dismantled complex blast injury, while 87% met the Joint Trauma System criteria for at risk for IFI, indicating that the criteria for a complex injury may not be an adequate predictor of IFI risk.

Recommendations: The 2011 TIDOS IFI Case Investigation determined that the critical elements for IFI management include key awareness of risk factors and common clinical findings, early diagnosis, appropriate handling of clinical specimens, aggressive surgical debridement, and use of empiric antifungal therapy where there is a strong clinical suspicion of an IFI. The findings of this report strengthen these recommendations and indicate that the PCR-based assay is most effective at detecting pathogenic fungi of the order Mucorales (particularly *Saksenaee* spp) when compared to conventional cultures, providing increased accuracy and timeliness to the diagnostic test strategy. Our recommendations include:

1. PCR-based assay should be utilized as a complement to histopathology and cultures to direct antifungal treatment
2. Specimens for PCR analysis should be collected following the completion of at least two surgical debridements after evacuation from the operational theater in order to avoid contamination and false-positive results
3. Initial coverage to include dual antifungals (i.e., liposomal Amphotericin B and voriconazole/posaconazole) should be directed toward the order Mucorales in patients with histopathological evidence of invasive filamentous fungi
4. Based on discordant findings between the panfungal and semi-nested PCRs, the relative performance of the two PCR-based methods, including testing of both traditional PCR-based methods and real-time PCR probes on both fresh and FFPE, should be the focus of future work

Table of Contents

EXECUTIVE SUMMARY	1
1. OBJECTIVE	5
2. BACKGROUND	5
2.1. Combat-Related IFIs.....	5
2.1.1. IFI Outbreak Investigation.....	5
2.1.2. IFI Epidemiology	6
2.1.3. Civilian Experience with Trauma-Related IFIs	7
2.2. Department of Defense Response to IFI Outbreak.....	7
2.2.1. Landstuhl Regional Medical Center Process Improvement Initiative	7
2.2.2. Joint Trauma System Clinical Practice Guideline	8
2.2.3. Department of Defense Clinical Research Initiatives.....	8
2.3. IFI Diagnostic Methods	9
2.3.1. Current Approach and Limitations	9
2.3.2. PCR-based Molecular Assays.....	10
3. INVESTIGATION METHODS	11
3.1. Study Design.....	11
3.2. Study Population.....	11
3.2.1. Subjects with Laboratory Evidence of Fungal Infection	12
3.2.2. Control Subjects.....	13
3.3. Tissue Specimens.....	13
3.4. Panfungal vs Semi-nested PCR Assay: Limited Assessment.....	14
3.5 Definitions	16
3.6. Statistical Methods.....	16
4. FINDINGS.....	17
4.1. Study Population and Characteristics	17
4.1.1 Dismounted Complex Blast Injuries.....	18
4.1.2. PCR-Based Assay Study Population	19
4.2. PCR-Based Molecular Assay.....	20
4.2.1. Pilot Study.....	20
4.2.2. Retrospective Analysis of Archived Tissue Specimens.....	21

4.2.3. Clinical Mycology Results (Culture-based) from Sites with Positive Histopathology	21
4.2.4. PCR-Based Assay Results from FFPE Tissue Specimens.....	22
4.2.5. Concordance of Mycology and PCR-based Assay	24
4.2.6. Panfungal vs Semi-nested PCR Assay: Limited Assessment	24
5. DISCUSSION AND RECOMMENDATIONS.....	25
5.1. Epidemiology of combat-related fungal wound infections	25
5.2. PCR identification of fungi from formalin fixed paraffin embedded tissue.....	27
5.3. Limitations	29
5.4. Adherence to DoD Recommendations	29
5.5. Recommendations.....	30
5.6. Follow-on Work.....	32
6. ACKNOWLEDGMENTS	32
7. REFERENCES	33
8. APPENDIX: SUPPORTING DOCUMENTATION.....	41

1. OBJECTIVE

The primary objective of the investigation was to develop and assess the performance characteristics of a PCR-based molecular assay designed for the rapid identification of filamentous fungi in formalin-fixed paraffin-embedded (FFPE) tissue specimens and fresh tissue specimens. Due to the cessation of combat operations the performance characteristics of the PCR-based assay on fresh tissue specimens could not be executed. The concordance of PCR findings with standard clinical mycology results were evaluated.

2. BACKGROUND

2.1. Combat-Related IFIs

2.1.1. IFI Outbreak Investigation

Following the transition of military personnel into Afghanistan in 2010, the increased incidence of trauma resulting from improvised explosive devices (IEDs) encountered while on foot patrol led the Joint Trauma System (JTS) to recognize a new injury pattern referred to as dismounted complex blast injuries (DCBI). A DCBI was characterized by an amputation of one lower extremity (at knee or above) with either an amputation or severe injury to the opposite lower extremity, as well as pelvic, abdominal, or urogenital injury.¹

Coinciding with the surge of service members into Afghanistan and the rising frequency of DCBIs, IFIs (due to filamentous fungi) emerged as serious infectious complications among combat casualties.^{2,3} The first reported cases were among United Kingdom (U.K.) military personnel injured in the Helmand Province.³ Dismounted blast injuries, extensive wounds or amputations heavily contaminated with debris, and >8 units of blood within 24 hours of injury were found to be common characteristics among the IFI patients. It was also noted that all of the patients with an IFI were injured in the southern region of Afghanistan, specifically the Helmand Province (the ‘Green Zone’). While surgical debridement was the primary treatment measure for managing the IFIs, antifungal prophylaxis (liposomal Amphotericin B and posaconazole)/preemptive treatment was also prescribed to patients who met criteria.³

As with the U.K. combat casualties, there was an outbreak of IFIs among U.S. wounded military personnel with blast trauma. Due to the high mortality and substantial morbidity associated with the disease (i.e., surgical amputations and/or amputation revisions, hemi-pelvectomies, and total hip disarticulations),^{2,4} there was a need to systemically assess the epidemiology of IFIs and examine clinical and long-term outcomes. In 2011, the Department of Defense (DoD)-Veterans Affairs (VA) Trauma Infectious Disease Outcomes Study (TIDOS)⁵ Team was tasked by the JTS leadership with conducting a case investigation to describe IFIs among combat casualties; assess clinical, microbiological, and histopathological characteristics of the IFI cases; review IFI-specific cases between June 2009 and December 2010. The findings demonstrated an increasing rate of IFI cases over the study period, particularly among patients admitted to the intensive care unit at

Landstuhl Regional Medical Center (LRMC; 8% in the fourth quarter of 2010). Recommendations from the case investigation included identification of IFI risk factors as a priority and incorporating the findings in pre-deployment training. Furthermore, defining what constitutes a suspicious wound was also determined to be critical and clinicians were advised to use the hallmark of recurrent wound necrosis, as well as the preliminary risk factors, to establish an IFI diagnosis as early as possible. Appropriate handling of clinical specimens to aid in diagnosis through coordination with infectious disease specialists, pathologists, and clinical microbiology staff was also encouraged. Regarding treatment, aggressive surgical debridement along with dual antifungal therapy (liposomal Amphotericin B and a triazole) when there was a strong suspicion of an IFI was recommended.

The IFI Case Investigation Technical Report was briefed to the Assistant Secretary of Defense (Health Affairs), Surgeons General, and COCOM Surgeons in June 2011.⁶ The findings and recommendations of the report led to standardized data capture related to fungal infections, including recurrent necrosis of wound tissue, histopathology, and antifungal therapy. Clinical aspects of early awareness, coupled with aggressive surgical debridement and adjunctive empiric antifungal chemotherapy were emphasized and later utilized in the JTS Clinical Practice Guideline (CPG; released November 2012).⁷

2.1.2. IFI Epidemiology

Following the initial IFI outbreak investigation, the TIDOS team extended the time period for the analyses out to August 2011, resulting in a population of 77 IFI cases (6.8% of 1133 combat casualties admitted to participating hospitals in the United States).⁴ When the data were analyzed by admitting unit at LRMC, the proportion of IFIs ranged from 0.2% among patients admitted to the ward to 11.7% for the intensive care unit. Blast injuries were predominant (96%), with the majority occurring while dismounted (90%), resulting in a median injury severity score of 21. In addition, patients frequently incurred a lower extremity amputation (78%) and required a median of 30 units of red blood cells within 24 hours of injury. Regarding outcomes, 19% of patients required either a hemipelvectomy or total hip disarticulation. Approximately, 8% of the patients died; however, all deaths were not directly attributable to the IFI.⁴ It is important to note that findings from the IFI outbreak investigation are specific to combat casualties wounded in support of operations in Afghanistan and may not represent the global spectrum of the disease.

Per the recommendations from the IFI Case Investigation Technical Report, data from 76 IFI patients were assessed in a case-control analysis to identify risk factors for the development of IFIs. Independent risk factors were determined to be sustaining a blast injury (odds ratio [OR]: 5.7; 95% confidence interval [CI]: 1.1-29.6) while dismounted (OR: 8.5; 95% CI: 1.2-59.8), having a traumatic above knee amputation (OR: 4.1; 95% CI: 1.3-12.7), and requiring at least 20 units of blood within 24 hours of injury (OR: 7.0; 95% CI: 2.5-19.7).⁸

To further examine the negative impact of IFIs on wound healing, clinical outcomes in patients with IFI and non-IFI wounds were compared. The findings demonstrated that IFI patients had a significantly longer time from injury to wound closure (median of 16 days versus 9 days), greater number of visits to the operating room, and a higher proportion of amputation revisions.⁹ Furthermore, a separate analysis examined the impact of mycology on wound healing. Among the 82 IFI wounds included in the analysis, 35% grew fungi from the order *Mucorales*, while 29% grew *Aspergillus* spp., and 21% grew *Fusarium* spp. Approximately a quarter of the IFI wounds grew at least two fungi. Of the wounds with *Aspergillus* spp. and *Fusarium* spp., five grew concomitant fungi from the order *Mucorales*. It was determined that wounds that grew fungi from the order *Mucorales* had a significantly longer time to wound closure compared to wounds with non-*Mucorales* growth (median of 17 days versus 13 days). While these findings confirm the pathogenicity of mucormycosis, it also demonstrated that wounds growing other fungi (e.g., *Aspergillus* spp. and *Fusarium* spp.) cannot be discounted.¹⁰

2.1.3. Civilian Experience with Trauma-Related IFIs

Trauma-related IFIs have also been reported among civilian populations, primarily associated with natural disasters, motor vehicle crashes, and agricultural accidents (**Table 1**).¹¹⁻³⁰ Fungi from the order *Mucorales* are most commonly attributed to the infections with *Rhizopus* spp.;^{13,18,27,29} however, reports of *Saksenaia* spp. are becoming more frequent.¹⁷ As with military patients, wound necrosis is the hallmark of the infection,^{12-14,29} and patients frequently require multiple surgeries, including amputations, to resolve the infection.^{12,13} Mortality among civilian patients is generally higher than what is reported for the military population, with rates as high as 38%.²⁵

In general, risk factors for the development of IFIs in civilian trauma patients has not been well examined. One analysis, not specific to trauma, found that independent risk factors for cutaneous mucormycosis were female sex, no underlying condition, prior surgery, and HIV infection.²² In another study involving IFI patients with injuries sustained in a tornado, penetrating wounds (OR: 8.8; 95% CI: 1.1-69.2) and occurrence of multiple wounds (OR: 2.0; 95% CI: 1.2-3.2) were found to be significantly associated with trauma-related IFI risk.²⁵

2.2. Department of Defense Response to IFI Outbreak

2.2.1. Landstuhl Regional Medical Center Process Improvement Initiative

Following recognition of the IFI outbreak, a local process improvement initiative (referred to as the 'Blast Protocol') was implemented at LRMC in February 2011. The objective of the Blast Protocol was to improve uniformity regarding the use of tissue-based diagnostics among at-risk blast trauma patients, thereby also improving use of antifungal therapy.³¹ Criteria used for the determination of patient risk stratification is in **Table 2**. Once classified as 'at risk for IFI', wound tissue specimens were collected during the first debridement at LRMC (generally the patient's 2nd or 3rd debridement) from each lower extremity amputation for histopathology and culture analysis.

Additional tissue specimens were collected at subsequent debridements at the discretion of the attending surgeon based on wound appearance and the patient's overall status.

In a TIDOS analysis, the effectiveness of the LRMC Blast Protocol was assessed by comparing patients who were admitted to LRMC before and after the implementation of the process improvement initiative. Findings showed that the Blast Protocol was successful as the time to IFI diagnosis was significantly reduced from a median of 9 days post-injury (IQR: 5-12) pre-Blast Protocol to 3 days post-injury (IQR: 2-5) post-Blast Protocol. Furthermore, antifungal therapy was also initiated earlier in the period of the Blast Protocol (median of 7 days post-injury; IQR: 5-10 versus a median of 15 days post-injury; IQR: 9-31 in the comparator group). A significantly lower proportion of cases with angioinvasion was also reported after the Blast Protocol was implemented (17% versus 48% in the earlier period), which may be due to earlier diagnosis and prompt treatment impeding progression of the disease. Nevertheless, there was no significant difference regarding duration of hospitalization or mortality between the groups.³¹

2.2.2. Joint Trauma System Clinical Practice Guideline

Findings from the TIDOS IFI Technical Report were utilized by the JTS to develop the first CPG related to the management of IFIs in war wounds, which was published in November 2012.⁷ The CPG focused on early detection of the infection, risk stratification, wound care, and timely medical treatment of suspected IFIs. Potential IFI risk factors were identified based on characteristics common to the 36 IFI patients in the TIDOS Outbreak Investigation, which included sustaining a dismounted blast injury; above knee amputation; extensive perineal, genitourinary, or rectal injury; and massive transfusion of >25 units of blood within 24 hours post-injury. Except for the perineal/genitourinary injuries, these characteristics were validated as risk factors in a subsequent TIDOS analysis (25 units of blood revised to 20 units of blood),⁸ as discussed in Section 2.1.2.

The CPG emphasized early and aggressive surgical debridements and advocated use of antifungal topical therapy. Dual antifungal therapy was recommended in cases of significant progressive tissue necrosis on two consecutive debridements (not counting the first two debridements commonly occurring in theater). Collection of tissue specimens at LRMC was also recommended for patients with at least three of the potential IFI risk factors. After admission in the United States, collection of tissue specimens was recommended for patients with at least three IFI risk factors, as well as a wound with appearance suspicious for necrotic wound infection.⁷

2.2.3. Department of Defense Clinical Research Initiatives

Clinical research related to IFIs became a Military Health System priority following the outbreak in 2010 in an effort to reduce the morbidity and mortality associated with the disease and improve patient prognosis. Over the years, multiple research initiatives have been undertaken at various DoD sites and laboratories (**Table 3**).

Clinical IFI research has included a comprehensive evaluation of IFI epidemiology, microbiology, and outcomes,^{2-4,8-10} as discussed in Section 2.1.2. Analyses have also assessed the utility of Dakin's solution,^{32,33} as well as diagnostic approaches.^{34,35} Findings from the clinical research initiatives were also utilized to refine the JTS CPG for the management of IFI in war wounds in 2016.³⁶

2.3. IFI Diagnostic Methods

2.3.1. Current Approach and Limitations

In order to improve outcomes among IFI patients (e.g., reducing need for amputations/amputation revisions), early identification of the causative fungus and subsequent timely intervention with appropriate antifungal therapy is needed. Presently, an optimal strategy for enabling earlier diagnosis of IFIs has not been determined as there are limitations related to the current techniques used for fungal identification. Although bacterial isolates are relatively easy to identify in a wound with the use of conventional culture techniques, cultures are often an insensitive (and time delayed) method for the identification of invasive fungal pathogens with frequent isolates representing wound contamination.³⁷⁻³⁹ Historically, the diagnosis of IFI utilizing conventional techniques has been problematic in immunocompromised patients (e.g., patients undergoing chemotherapy or bone marrow transplantation) and the yield of fungal cultures in these settings has been low.^{39,40} Similarly, among 19 burn patients with histopathological evidence of fungal wound infections examined at the Brooke Army Medical Center (BAMC), only 9 (47%) exhibited corresponding growth on culture.³⁸ It is also well known that many fungal infections (e.g., pulmonary aspergillosis) rarely yield live organisms after culture.³⁷ Furthermore, several factors impact the yield of culture results from biopsy specimens. First, the empiric use of antifungals may reduce the sensitivity of cultures. Second, pretreatment of tissue samples (grinding) prior to inoculation of culture media may disrupt fungal hyphae and reduce growth. Lastly, culture conditions in the laboratory may not be optimal for the growth of fungi as they fail to mimic conditions of growth in the human tissue (e.g., low oxygen tension often found in necrotic tissue), thereby reducing the likelihood of obtaining positive culture results.^{39,41} The results of cultures are also often delayed due to the requirement of the fungus to produce distinguishing structures in culture, some of which may take weeks. An internal review of fungal wound cultures performed at National Naval Medical Center (NNMC) indicated that it frequently took one to two weeks to receive results, thus limiting their ability to provide guidance for initial treatment choices. Morphologic identification of less common fungi also requires considerable expertise, which is rarely available.

Histopathological identification of fungi in tissue is the gold standard for the rapid diagnosis of IFI; however, this method possesses limitations as it does not allow for species level (and sometimes genus level) identification. There is also potential for sampling error. Among the 77 combat casualties diagnosed with IFIs between 2009 and 2011, histopathological evidence of fungal elements was observed in 54 (70%) of the patients.⁴ Thus, reliance on histopathological evidence alone to diagnose an IFI would have resulted in the failure to identify approximately a

third of the cases. Furthermore, misidentification, false-negatives, and false-positives may occur with histopathology. The utility of Gomori methenamine silver (GMS) and periodic acid-Schiff (PAS) special stains in diagnosing IFIs was examined using 74 tissue specimens from 28 IFI patients. The stains were 84% concordant with identifying fungal elements; however, the false-negative rates between GMS and PAS differed (15% versus 44%, respectively).³⁵

Due to the challenges inherent with identifying fungal organisms via routine culture and on histopathological exam, as well as the concern that using a single antifungal agent may not adequately treat the variety of potential fungi in a wound, patients with suspected IFI are often prescribed dual antifungal therapy with both a triazole (voriconazole or posaconazole) and liposomal Amphotericin B. Antifungals are known for potential adverse effects. In particular, use of liposomal Amphotericin B may result in renal failure, significant infusion reactions, anemia, hypokalemia and hypomagnesaemia, while voriconazole has been associated with significant hepatotoxicity, visual hallucinations and photosensitivity.^{42,43} In addition, as voriconazole is metabolized through the cytochrome P450 system (CYP450), there is the potential for significant drug-drug interaction, which may further complicate the delivery of care for these patients.⁴⁴

2.3.2. PCR-based Molecular Assays

Given that delayed initiation of antifungals is associated with a worsened outcome,^{45,46} diagnostic tests that can rapidly and accurately identify subjects with an IFI are essential. Molecular-based methods involving DNA sequencing offer a high degree of precision related to species identification. Since PCR is a molecular-based diagnostic platform, it is culture-independent and, therefore, does not require the growth of live organisms, resulting in turnaround times as fast as a few hours. In general, molecular methods for the detection of fungi have been studied primarily in immunocompromised subjects,⁴⁷⁻⁵⁶ particularly related to the identification of *Aspergillus* DNA.⁵⁷⁻⁶⁴ In recent years, PCR assays targeted toward the order Mucorales have also been developed.^{50,65-67} One semi-nested PCR method targeted for rDNA of order Mucorales fungi had 86% sensitivity, demonstrating that it was more sensitive for fungal detection than conventional tissue and blood cultures.⁶⁵ Another multiplex real-time PCR was designed to detect the clinically relevant fungi, *Rhizopus oryzae*, *Rhizopus microsporus*, and *Mucor* spp. The assay demonstrated 100% sensitivity with samples containing the species for which it was designed; however, other fungi from the order Mucorales were not detected.⁶⁶ Rapid (within 6 hours) fungal identification has also been shown with unfixed tissue specimens from patients with histopathological fungal evidence using PCR/electrospray-ionization mass spectrometry.⁵⁵ In one prospective analysis, the integration of the PCR assay into the Mycoses Study Group criteria for IFI diagnosis resulted in an increased rate of diagnosis of proven IFI (71% compared to 53% without use of PCR).⁶⁸

Recent analyses have shown that fungal DNA can also be efficiently amplified by PCR from FFPE tissue samples (**Table 4**).^{51,52,69-79} The results of analyses using different PCR assays targeting either internal transcribed spacer (ITS) units or ribosomal DNA have had varying success with

sensitivities ranging from 38% to 89%. Specificity was only detailed in three analyses, but all reported 100%.

Overall, a PCR-based assay appears to have the capability of providing the desired improvements in turnaround time, precision, sensitivity, and specificity in fungal detection and identification relative to the current culture-based techniques. Therefore, our objective was to develop and assess the performance characteristics of a PCR-based molecular assay for the rapid identification of non-*Candida* fungi in fixed tissue specimens obtained during surgical debridement and to assess the concordance of histopathological and microbiological results with those obtained by PCR-based methods.

3. INVESTIGATION METHODS

3.1. Study Design

The utility of the PCR-based assay was retrospectively assessed using archived FFPE tissue samples from subjects with IFIs. Information utilized for this analysis was collected through the IFI Molecular Diagnostics protocol (IDCRP-077) and TIDOS (IDCRP-024),⁵ which is an observational, multicenter study focused on examining the short- and long-term outcomes of trauma-related infections among wounded military personnel. The IFI Molecular Diagnostics protocol was also designed to include prospective tissue specimen collection; however, there were few admissions to the participating U.S. hospitals following protocol approval due to the cessation of combat operations in Afghanistan. These low numbers precluded conducting the analysis of prospectively collected tissue specimens. Presently, the prospective study remains open for the potential of future conflicts.

This retrospective investigation was approved by the Infectious Disease Institutional Review Board of the Uniformed Services University of the Health Sciences. As the use of previously collected tissue would not adversely affect the rights and welfare of the subjects, a waiver of consent was obtained. This study did not exclude women and is restricted to adult DoD beneficiaries.

3.2. Study Population

Subjects who were included during the TIDOS data collection period were considered eligible for inclusion in the retrospective study if they were injured in the Afghanistan theater and admitted to a participating U.S. military hospital after transition through LRMCM between June 2009 and December 2014; and were suspected of having an IFI. The participating military hospitals were Walter Reed National Military Medical Center (WRNMMC; formerly NNMC and Walter Reed Army Medical Center), in Bethesda, Maryland, and BAMC, in San Antonio, Texas.

3.2.1. Subjects with Laboratory Evidence of Fungal Infection

The TIDOS database was searched for subjects with suspected IFIs based on meeting at least one of the following criteria: 1) Presence of a positive fungal culture and/or histopathological evidence of infection from wound tissue specimens following an initial adequate debridement; 2) Receipt of a systemic Amphotericin B formulation, voriconazole, and/or posaconazole for at least five days; and 3) Presence of epidemiological characteristics previously associated with a high risk of IFI (i.e., receipt of massive blood transfusions, dismounted blast injuries, and traumatic above knee amputations).⁸ Subjects with positive cultures from sputum, tracheal aspirate, or other sites unrelated to the traumatic wound were excluded. Charts for the subjects who met at least one of the criterion were reviewed for indications of wound necrosis after serial surgical debridements.

Once a suspected IFI was identified through retrospective review, the DoD Trauma Registry,⁸⁰ supplemental TIDOS infectious disease module,⁵ and the subject's medical records were examined to ascertain the mechanism of injury, clinical history, receipt of systemic antifungals, results of culture and histopathology, and operation reports. To be classified as an IFI, the diagnostic criteria required a traumatic wound with recurrent tissue necrosis following two serial irrigation and surgical debridements at LRMC or participating U.S. hospitals to exclude transient surface contamination, as well as laboratory evidence of a fungal infection (non-*Candida* filamentous fungi) at the time of or after the observed necrosis. The IFI subjects were categorized as having proven, probable, or possible IFI utilizing modified criteria of the Mycoses Study Group established by the TIDOS Investigative Team.⁴ A proven IFI was defined as histopathological evidence of fungal angioinvasion of wound tissue, regardless of culture confirmation. To meet the definition of a probable IFI, the subjects was required to have histology with fungal invasion in non-necrotic tissue without observed angioinvasion. A possible IFI had fungi isolated from tissue culture with either negative (no documented tissue invasion) histological findings or histology was not sent for examination.

Subjects with evidence who failed to meet the IFI diagnostic criteria (either because they did not have evidence of ongoing necrosis or had cultures and/ or histopathology collected prior to their debridements) were classified as High Suspicion if they had documented evidence of a soft-tissue infection that was attributable to the fungus and received antifungal therapy for at least 10 days. Wounds were also included in the High Suspicion groups if they received antifungals for <10 days and they had an amputation at the site of infection within 10 days of injury or died. Lastly, subjects with wounds failing to meet either the criteria of an IFI or High Suspicion of IFI were classified as Low Suspicion. To be clear, the Low Suspicion wounds did have tissue diagnostic evaluations and laboratory evidence of fungal infection/exposure, so do not generally represent 'low risk of IFI' wounds.

Subjects classified as an IFI, High Suspicion, or Low Suspicion were considered for inclusion in the retrospective analysis of archived tissue specimens if they had surgical pathology specimen with positive histopathology available for analysis.

3.2.2. Control Subjects

Subjects not meeting the above criteria for IFI, High Suspicion, or Low Suspicion were considered for inclusion in the control population. The negative controls for this study included subjects who had similar epidemiologic risk factors (such as blast injuries, receipt of massive transfusion) but did not demonstrate any laboratory evidence of fungal infections (such as positive cultures, positive histopathology, or ongoing tissue necrosis) or have evidence of a bacterial infection on histopathology, but had tissue samples available for analysis.

In addition, we also submitted tissue specimens from subjects who were not war-wounded as a non-trauma comparator population not at risk.

3.3. Tissue Specimens

In order to test the feasibility of the retrospective analysis, a pilot study was conducted using 30 surgical pathology FFPE specimens from nine subjects with an IFI provided to two independent laboratories for blinded analysis (LADR GmbH MVZ Dr. Kramer & Kollegen, Germany [a commercial laboratory]; and UTHSCSA). Based on the findings (see **Section 4.2.1**), we conducted the retrospective analysis reported herein of archived tissue specimens from subjects with positive histopathology (angioinvasion and nonvascular tissue invasion).

Surgical pathology specimens identified at the wound level were procured by the Pathology service at WRNMMC, BAMC, and LRMC. Tissue blocks were loaded in an aseptic fashion. A sterile microtome was used to section each tissue block. To reduce contamination, the first 5 micron section of the block was discarded. Sections (5-20 μm) were cut and shipped overnight in sterile cryovials to the two testing sites. Blinded specimens were submitted to University of Texas Health Sciences Center San Antonio, Texas (UTHSCSA) for evaluation. The PCR-based assays were developed as part of a contractual agreement with the Advanced Nucleic Acid Core Facility at the UTHSCSA. Fungal DNA was extracted from formalin-fixed tissue samples and paraffin impregnated tissue blocks utilizing sterile techniques, PCR-amplified, and then sequenced using previously described methods.^{70-72,81} For DNA extraction, five paraffin sections were transferred to a 2.0 ml screw cap tube (Sarstedt, Numbrecht, Germany) and pelleted briefly in a microfuge at low speed. Four hundred microliters of incubation buffer containing 2 mg/ml Proteinase K (Maxwell 16 FFPE Tissue LEV DNA purification kit, Promega, Inc, Madison, WI) were added to each specimen followed by incubation of the samples in a 70°C water bath overnight with periodic vortexing. After incubation, 100 μl (vol/vol) 0.5 mm glass beads (BioSpec Products, Inc., Bartlesville, OK) were added to each tube. Tubes were agitated on a bead beater (BioSpec) for one minute at maximum speed and then spun in a microfuge at maximum speed for one minute.

Supernatants were transferred to a new 2.0 ml screw cap tube, taking care not to carry over beads or debris. Two volumes of Maxwell 16 FFPE Tissue LEV DNA purification kit lysis buffer were added to each tube, which were mixed by briefly vortexing. Samples were stored overnight at room temperature, but not refrigerated, or processed immediately. The DNA was purified from the samples by using the Maxwell 16 Automated DNA extraction instrument (Promega), according to manufacturer's instructions. After purification, samples were measured for DNA content by NanoDrop spectrophotometer (ThermoFisher, Grand Island, NY).

For fungal identification at UTHSCSA, template DNA was amplified by PCR using the primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') to amplify the ITS2 region in the fungal ribosomal DNA locus as previously described.⁸² Amplicons were sequences with the same primers in the Advanced Nucleic Acids Core Facility at UTHSCSA. Sequences were then used for a BLAST search of GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Identifications were based on BLAST searches with identities $\geq 97\%$. Additional PCR and sequencing was performed when needed for species confirmation or if BLAST results yielded low identities.

3.4. Panfungal vs Semi-nested PCR Assay: Limited Assessment

The PCR-based diagnostic method was evaluated to determine if it had an acceptable level of concordance with the clinical diagnosis and conventional methods of identification (e.g., culture and histopathology). To compare PCR assay reproducibility, 18% of the blinded specimens were also sent to 'LADR GmbH, Medizinisches Versorgungszentrum Dr. Kramer & Kollegen' in Germany and analyzed using an alternate method.^{67,78} To enhance the sensitivity of a published *Aspergillus fumigatus* PCR, a third primer was added to run a semi-nested PCR assay, as previously described.⁶⁷ P2 (5'-CTT TGG TTG CGG GTT TAG GGA TT-3') and Asp2 (5'-GGG AGT TCA AAT CTC CCT TGG G-3') are complimentary to nucleotide positions 938–916 and 738–759, respectively, of *A. fumigatus* mitochondrial transfer RNA (GenBank accession number L37095; <https://www.ncbi.nlm.nih.gov/nucore/L37095>).⁸³ The product of the semi-nested reaction using P1 (5'-GAA AGG TCA GGT GTT CGA GTC AC-3') and P2 is 135 bp long. A semi-nested PCR targeting the V4 and V5 region of the 18S ribosomal DNA of fungi belonging to the order Mucorales was used to detect the DNA of etiologic agents responsible for causing mucormycosis. The outer primers ZM1 (5'-ATT ACC ATG AGC AAA TCA GA-3') and ZM2 (5'-TCC GTC AAT TCC TTT AAG TTT C-3') are complementary to nucleotide positions 711 to 730 and 1117 to 1096 of *Rhizopus* (GenBank accession number AF113440; <https://www.ncbi.nlm.nih.gov/nucore/AF113440>), respectively. Products of the semi nested reaction using primers ZM1 and ZM3 (5'-CAA TCC AAG AAT TTC ACC TCT AG-3') are 175 to 177 bp long, depending on the genus within the order Mucorales.⁶⁷ For *Fusarium* spp., a semi-nested PCR assay utilized FUS I (P58SL) and FUS II (P28SL) primers (5'-AGT ATT CTG GCG GGC ATG CCT GT-3' and 5'-ACA AAT TAC AAC TCG GGC CCG AGA-3', respectively) to amplify a 357 bp DNA sequence within the ITS/28s region of *Fusarium* spp.⁸⁴ The FUS I primer

is complementary to position 329-351 and FUS II is complementary to position 685-662 (Genbank accession number L36634, gi:973988; <https://www.ncbi.nlm.nih.gov/nuccore/L36634>). To increase sensitivity, we constructed a FUS III primer (5'-CCG TTA CTG AGG CAA TCC CTG TT-3'). The semi-nested product of FUS I and FUS III is 296 bp long. The PCR conditions are identical to the ones used for *Aspergillus* and *Mucorales* specific PCRs. The reaction mixtures of the primary PCRs consisted of 10 µl previously extracted DNA in a total volume of 50 µl, with final concentrations of 10mM Tris/HCl (pH 8.3), 50mM KCl, and 2.5mM MgCl₂ (10× Perkin-Elmer buffer II plus MgCl₂ solution; Roche Molecular Systems, Branchburg, New Jersey, USA); 1µM of each primer of the outer primer sets (ZM1/ZM2 and P2/Asp2, respectively; Roth, Karlsruhe, Germany); 1.5 U of AmpliTaq DNA polymerase (Roche); and 100µM of each dNTP (Promega, Madison, Wisconsin, USA). Identical reaction mixtures were used for the nested PCRs except that 1 µl of the first reaction, 50µM of each dNTP, and 1µM of each inner primer (ZM1/ZM3 and P1/P2) were used. All reaction mixtures were thermally cycled once at 94°C for five minutes, 35 times at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for one minute, and then once at 72°C for five minutes. To validate the presence of amplifiable DNA and absence of inhibitory substances, a PCR was performed using the primer set G1 (5'-GAA GAG CCA AGG ACA GGT AC-3') and G2 (5'-CAA CTT CAT CCA CGT TCA CC-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 above except that 5 µl DNA extract and 3mM MgCl₂ were used, and the extension time was reduced from one minute to 45 seconds. When the result was negative, DNA extraction was repeated if enough material was available. All PCRs were run in a Primus PCR thermocycler Tc 9600 (MWG Biotech, Ebersberg, Germany). 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.⁸⁵ 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. The nested PCR products were sequenced by a semi-automated system (Applied Biosystems Division, Perkin-Elmer Biosystems, Foster City, California, USA) and sequences were used for a BLAST search of the GenBank database.⁸⁵ A PCR assay was considered positive if the product obtained from the *Aspergillus* PCR was identical to an *Aspergillus* sequence in GenBank. If the product amplified by *Mucorales* PCR showed 100% homology to an 18S rDNA sequence of a member of the order *Mucorales*, and if the product of the *Fusarium* PCR showed 100% sequence identity to a member of the genus *Fusarium*.

Results from UTHSCSA and the laboratory in Germany were compared.

3.5 Definitions

All patients in the analysis were restricted to those who sustained open extremity injuries in Operation Enduring Freedom (Afghanistan theater) and transferred to a participating hospital in the United States.

Subjects included in retrospective analysis of archived surgical pathology FFPE specimens

Positive Histopathology	Subjects with evidence of angioinvasion or nonvascular tissue invasion with fungal elements.
Controls	Subjects with tissue specimen collection and no evidence of a fungal infection (by histopathology or culture) or a bacterial infection (by histopathology).

Subjects with laboratory evidence (culture/histopathology) of fungal infection

IFI	A traumatic wound with recurrent tissue necrosis following two serial irrigation and surgical debridements to exclude transient surface contamination, as well as laboratory evidence of a fungal infection (non- <i>Candida</i> filamentous fungi) at the time of or after the observed necrosis.
<i>Proven IFI</i>	Angioinvasion of wound tissue
<i>Probable IFI</i>	Nonvascular tissue invasion
<i>Possible IFI</i>	Fungi identified on cultures; histopathology was either negative or not sent
High Suspicion	Laboratory evidence of fungal infection along with signs and symptoms of an infection that were attributed to the fungus at the wound site and receipt of antifungals for ≥ 10 days. Subjects with < 10 of antifungal treatment are also included if they had an amputation or death within 10 days.
Low Suspicion	Laboratory fungal evidence but did not meet IFI or High Suspicion criteria

3.6. Statistical Methods

Patient characteristics were compared using Fisher's exact test or Chi-square (as appropriate) for categorical variables and Wilcoxon rank-sum test for continuous variables. P-values of < 0.05 were considered statistically significant.

For the assessment of the culture and PCR-based assay findings for the surgical pathology FFPE specimens, the analysis was done on the injury site level. If an injury site contributed multiple specimens to the analysis, the findings were collapsed for the site. For this analysis, specimens where the only fungus identified were common skin and environmental contaminants namely *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Malassezia globosa*, *Malassezia nana*, *Malassezia restricta*, *Trichosporon asahii*, and *Yarrowia lipolytica* were analyzed as negatives.

Measures of concordance/agreement between conventional culture mycology and PCR findings were calculated using Cohen's kappa coefficient with 95% confidence intervals. The results were considered concordant if there was a match at the genus level. Concordance of the PCR findings and histopathology were calculated using Cohen's kappa coefficient with 95% confidence intervals and McNemar's test statistic. Diagnostic performance parameters (e.g., sensitivity, specificity, and negative predictive value) assessing the PCR-based assay against histopathology were calculated as previously described.⁸⁶ Measures of concordance between the different PCR-based assays utilized by UTHSCSA and LADR were assessed using Cohen's kappa coefficient with 95% confidence intervals. Findings were considered concordant if there was a match at the genus level; however, specimens identified as *Apophysomyces elegans* were included with *Saksenaea* spp. as LADR was unable to differentiate between the two organisms.

4. FINDINGS

4.1. Study Population and Characteristics

Among 720 combat casualties with open extremity wounds admitted to participating U.S. military hospitals between 2009 and 2014 who had operative tissue specimens collected for histopathology and/or wound culture at any level of care (i.e., theater, LPMC, or U.S. hospitals) during their initial hospitalization, 246 (34%) had laboratory evidence of a fungal infection (**Figure 1**). Ninety-four (38%) patients met criteria for an IFI (143 wound sites), based on occurrence of laboratory evidence combined with recurrent necrosis. Sixty-one (25%) patients with laboratory fungal evidence were classified as High Suspicion of fungal infection (120 wound sites), while 91 (37%) patients were categorized as Low Suspicion of fungal infection (150 wound sites).

The patients in the three groups were predominantly injured via a blast mechanism while on foot patrol (95% of the patients with data available), resulting in a high proportion of traumatic amputations (68%, 78%, and 80% for IFI, High Suspicion, and Low Suspicion, respectively; **Table 5**). Although the difference between the groups is not statistically significant, there is a trend toward significance when comparing the proportion of traumatic amputations between the IFI and Low Suspicion groups ($p=0.060$). Injury severity was also high among all three groups; however, IFI patients had a higher proportion of critical injury severity scores (ISS) compared to High Suspicion (93% versus 85% with $ISS \geq 26$, respectively) and Low Suspicion group (93% versus

82%). The median ISS among IFI patients was also significantly higher compared to Low Suspicion patients (40 versus 33). A significantly higher proportion of IFI patients also received large-volume blood transfusion within 24 hours post-injury compared to both the High Suspicion and Low Suspicion groups (median units: 31 versus 21 and 17, respectively). Furthermore, there was a significant difference in the distribution of IFI patients requiring mechanical ventilation compared to the Low Suspicion patients ($p<0.001$).

There were also significant differences between the High Suspicion and Low Suspicion groups. In particular, the High Suspicion had a higher median ISS ($p=0.017$) and sequential organ failure assessment (SOFA) scores at LPMC and U.S. hospitals ($p=0.003$ and $p<0.001$, respectively), compared to Low Suspicion patients. There was also a significant difference in the distribution of subjects receiving mechanical ventilation between the two groups ($p=0.010$). Nonetheless, there was no statistically significant difference regarding blood volume and traumatic amputations when the High Suspicion patients were compared to the Low Suspicion group ($p=0.055$ and $p=0.818$, respectively).

When clinical culture mycology was examined on a site level, there were differences between the groups (**Figure 2**). In particular, the 143 IFI sites had a higher proportion of fungi from the order Mucorales on culture (39% of IFI sites vs 22% and 9% of the 120 High and 150 Low Suspicion sites, respectively). Furthermore, other fungi (i.e., fungi other than order Mucorales, *Aspergillus* spp., and *Fusarium* spp.) were more commonly isolated from Low Suspicion sites (46% versus 13% and 23% for IFI and High Suspicion sites).

4.1.1 Dismounted Complex Blast Injuries

Among the combat casualties admitted to hospitals in the United States, 162 (8% of 1932; 23% of 720 subjects with tissue specimens/wound cultures collected for analysis) met the full definition of a DCBI (i.e., dismounted blast injury resulting in an amputation at or above knee with either an amputation or severe injury to the opposite lower extremity, as well as pelvic, abdominal, or urogenital injury). The 162 patients were assessed for laboratory evidence of a fungal infection. Specifically, 49 (30%) met the criteria for an IFI, 32 (20%) were classified as High Suspicion, and 38 (23%) were classified as Low Suspicion. The remaining 43 (27%) subjects did not have any laboratory evidence of a fungal infection (a majority of these subjects [$N=35$] had specimens collected for histopathology/fungal cultures and the results were negative; 8 had wound cultures collected). When the IFI and High Suspicion subjects ($N=81$) were compared to subjects who were classified as Low Suspicion or did not have any laboratory fungal evidence ($N=81$), there were significant differences between the groups (**Table 6**). Specifically, the DCBI patients with an IFI or High Suspicion had significantly greater injury severity (median ISS: 38 vs 33; $p=0.001$) and had more large-volume blood transfusions within 24 hours (median units: 30 vs 20; $p<0.001$). In addition, distribution of subjects who required mechanical ventilation was significantly different ($p=0.001$). Furthermore, the IFI and High Suspicion DCBI patients had higher SOFA scores at

both LRMC and the U.S. hospitals ($p<0.001$) and significantly more had colostomies (33% vs 15%; $p=0.006$).

The remaining IFI ($N=45$) and High Suspicion ($N=29$) subjects who did not meet the full definition of a DCBI were examined (**Table 6**). Despite not meeting the full criteria for a complex injury, the majority of these patients sustained a blast injury (97%) while dismounted (86%), were severely injured (78% with an ISS ≥ 26), and required a median of 24 units of blood within 24 hours of injury. There was no significant difference in SOFA scores at LRMC (median: 10 vs 9; $p=0.853$) and U.S. hospitals (median: 6 vs 7; $p=0.581$), or use of mechanical ventilation ($p=0.760$) when compared to the IFI and High Suspicion patients who met the criteria for a DCBI.

If the 24 subjects with missing mounted status were excluded, 62% of the 131 IFI and High Suspicion of IFI subjects met the full criteria for a DCBI. In contrast, 87% (114 of 131 subjects) met the criteria for at risk for IFI based on the 2012 JTS CPG (Note: while 2012 JTS CPG lists >25 units of blood within 24 hours post-injury, additional risk factor analysis identified >20 units of blood as an independent IFI predictor and that volume was utilized in the calculation).^{7,8} These findings suggest while sustaining a dismounted blast and receiving large-volume blood transfusions are good predictors for IFI risk, the criteria of complex injury is not as reliable.

4.1.2. PCR-Based Assay Study Population

For the retrospective analysis of archived surgical pathology FFPE specimens, 64 subjects with positive histopathological evidence of a fungal infection (35 Proven, 22 Probable, 1 Possible, 5 High Suspicion, and 1 Low Suspicion) were included in the study population (**Figure 1**).

All of the specimens from histopathology positive subjects were collected from hospitals in the United States. The 64 subjects had a total of 95 injury sites, of which 41 subjects contributed one site, 17 subjects contributed two sites, 4 subjects contributed three sites, and 2 subjects contributed four sites. A total of 171 specimens were collected from the 95 injury sites with positive histopathology (47 sites had only one specimen collected, 29 had two specimens, 14 had three specimens, 2 had four specimens, 2 had five specimens, and 1 had six specimens). Among the 48 sites with multiple specimens collected, 37 (78%) had the specimens collected within one week of the first specimen. Eleven injury sites had a duration of ≥ 7 days between collection of the first and last specimen. When the first and last specimens were compared, the findings were analogous for five sites (three sites had negative findings on both specimens, while two sites were positive for fungal DNA with both specimens). Among the sites with discordant findings between the first and last specimens, five sites were positive for fungal DNA with the first specimen and negative on the last specimen, while one site was negative on the first specimen and positive for fungal DNA with the last specimen. Findings from multiple specimens for a single injury site were collapsed for analysis. Fungal cultures from the injury sites involved in the analysis were collected

at either LRMC or the U.S. hospitals for 87 (92% of 95) of injury sites for the subjects with positive histopathology.

Specimens from 148 control subjects (193 injury sites) who did not have any laboratory evidence of a fungal infection (culture or histopathology) were submitted for analysis. Forty-six of the subjects did not meet the study population criteria (i.e., combat-related open wounds sustained in Afghanistan theater, transfer to a participating U.S. hospital, and surgical pathology specimen collected from an extremity wound) and were excluded from the analysis. The resultant control population includes 102 control subjects with 118 injury sites (**Figure 3**). Among the control subjects, 87 contributed one injury site, 14 had two sites, and one had three sites. A total of 128 tissue specimens were collected (110 injury sites with one specimen, 7 sites with 2 specimens, and 1 site with 4 specimens).

All subjects in both the positive histopathology and control groups were male. The subjects with positive histopathology had a significantly greater proportion of blast injuries (97% vs 80%; $p=0.002$), higher injury severity (median ISS: 42 vs 26; $p<0.001$), and required more large-volume blood transfusions within 24 hours of injury (median units: 32 vs 10; $p<0.001$) compared to the control subjects (**Table 7**). In addition, the patients with positive histopathology had a greater proportion of traumatic above knee amputations (59% versus 21%; $p<0.001$), as well as perineal, genitourinary, and/or abdominal injuries (95% vs 62%; $p<0.001$).

4.2. PCR-Based Molecular Assay

4.2.1. Pilot Study

Fungal DNA extraction and preparation was successful in the pilot study using 30 surgical pathology FFPE specimens, except in one specimen at UTHSCSA. Compared to results on histopathology, of the specimens with tissue invasion, 100% at LADR and 95% at UTHSCSA (due to the extraction failure) identified filamentous fungal DNA, respectively. In 22 specimens with concomitant microbiological findings that could be assessed, the findings were 78% concordant in both laboratories at the genus level. Furthermore, PCR was able to provide genus and species identification with seven specimens where culture results were described as ‘mold not otherwise specified’ or ‘zygomycetes’. Six additional specimens from patients that did have combat trauma and were not suspected of having an IFI were assessed as controls. Five specimens were negative and one specimen showed a weak PCR band likely for *Malassezia restricta*, which is a common skin fungus. Based on these findings, the study proceeded to the full retrospective analysis.

4.2.2. Retrospective Analysis of Archived Tissue Specimens

Specimens from 95 injury sites with positive histopathology were examined. For examination of histopathology and culture findings, injury sites with positive histopathology were restricted to the 87 sites that also had a culture from the same site collected for analysis (8 injury sites without cultures excluded). Nineteen (22% of 87) sites had negative fungal cultures despite evidence of fungal angioinvasion or nonvascular tissue invasion being observed (**Table 8**). When restricted to cultures with fungal growth (N=68), 46% grew fungi from the order Mucorales. Although not included in the analysis, it should be noted that cultures from 11 sites also grew *Candida* spp.

The overall frequency of PCR-based fungal detection from sites with positive histopathology were examined based on whether they had angioinvasion (N=48) or nonvascular tissue invasion (N=47; **Table 9**). For sites with angioinvasion, a higher proportion of fungi from the order Mucorales was detected (75% versus 21% in sites with nonvascular tissue invasion). *Saksenaea* spp. contributed the highest proportion of order Mucorales fungi and was present in specimens from 26 (54%) sites with angioinvasion and 4 (9%) sites with nonvascular tissue invasion. *Aspergillus* spp. were identified in specimens from 15% of sites with angioinvasion and 9% of sites with nonvascular tissue invasion with *A. flavus* being predominant. A low proportion of *Fusarium* spp. was also identified in specimens from 4% and 9% of sites with angioinvasion and nonvascular tissue invasion, respectively.

4.2.3. Clinical Mycology Results (Culture-based) from Sites with Positive Histopathology

Sixty-eight (78% culture sensitivity of 87) injury sites with positive histopathology had cultures positive for growth of filamentous fungi (**Tables 8 and 10**); however, 5 sites grew fungi that were not identifiable at a genus level (e.g., *Mycelia sterilia* and fungi classified as only zygomycetes). Fungi from the order Mucorales were detected in cultures from 31 (36%) injury sites (8 sites with *Saksenaea* spp.), while *Aspergillus* spp. and *Fusarium* spp. were identified in cultures from 24 (28%) and 15 (17%) sites, respectively. As the control population was selected based on negative histopathology, performance characteristics (i.e., specificity, positive predictive value, false positive rates, and positive likelihood ratio) cannot be calculated for culture data nor compared with PCR findings. When restricted to 44 sites with evidence of angioinvasion (4 sites without a culture collected were excluded), 37 (84%) sites detected filamentous fungi, with cultures from 20 sites identifying fungi from the order Mucorales (**Tables 8 and 11**).

When restricted to specimens from injury sites with cultures collected only at U.S. hospitals (total of 86 sites with positive histopathology available), 62 (72% culture sensitivity with positive histopathology) sites had cultures with growth of filamentous fungi, while 24 (28%) sites had cultures with no growth (**Table 10**). Genus-level fungi were identified in cultures from 57 sites (66%). Fungi from the order Mucorales were detected in cultures from 29 (34%) injury sites (8 sites with *Saksenaea* spp.), while *Aspergillus* spp. and *Fusarium* spp. were identified in cultures

from 21 (24%) and 13 (15%) sites, respectively. Among cultures from 44 injury sites with angioinvasion, 37 (84%) had growth of filamentous fungi with 20 (46%) identifying order Mucorales (**Table 11**).

Timing of the cultures was also examined (**Table 10**). A total of 77 sites with positive histopathology also had cultures collected from the injury sites within 24 hours of the histopathological specimen, of which 55 (71% culture sensitivity) had growth of filamentous fungi. In particular, fungi from the order Mucorales were detected in cultures from 25 (33% of 77; 45% of 55 sites with fungal growth) injury sites, while *Aspergillus* spp. and *Fusarium* spp. were identified in cultures from 14 (18%) and 8 (10%) sites, respectively. For 41 sites with angioinvasion, 35 (85%) were positive for fungi growth with 18 (44%) identifying fungi from the order Mucorales (**Table 11**).

4.2.4. PCR-Based Assay Results from FFPE Tissue Specimens

A total of 363 FFPE specimens were sent for laboratory analysis; however, 64 specimens from control subjects who did not meet the study population criteria were excluded, resulting in 299 FFPE specimens eligible for analysis. Nine specimens from non-trauma comparator subjects were also analyzed as a quality assurance measure of the assay and the results are reported separately in **Section 4.2.5**. The extraction and sample preparation were successful for all specimens (including two specimens with extraction failures in the pilot study).

When the PCR findings were restricted to specimens from sites where cultures were also collected from either LRMC or U.S. hospitals (N=87), the assay was negative with specimens from 30 (35%) sites that had histopathological evidence of a fungal infection, while fungal DNA was identified with specimens from 57 (66%) sites (**Table 10**). Fungi from the order Mucorales were identified in specimens from 43 (49%) sites, which is higher than the 36% identified on cultures. *Aspergillus* spp. DNA was detected in specimens from 12 (14%) injury sites, while *Fusarium* spp. DNA was detected in 6 (7%) injury sites. When restricted to sites with angioinvasion and a culture collected from the injury site (N=44), specimens from 37 (84%) sites detected fungal DNA with the highest proportion identifying fungi from the order Mucorales (33 sites; 75%; **Table 11**).

Examination of the PCR findings from injury sites with cultures collected only at U.S. hospitals (total of 86 sites with positive histopathology available and 44 sites with angioinvasion), the proportion of growth of filamentous fungi remained the same (**Tables 10 and 11**). When the timing of cultures was restricted to those collected from the injury site within 24 hours of histopathological specimens (N=77), 48 (62%) injury sites had specimens with filamentous fungi DNA detected (**Table 10**). Fungi from the order Mucorales were detected in specimens from 37 (48% of 77; 77% of 48 sites with fungal DNA identified) injury sites (26 sites with *Saksenaee* spp.), while *Aspergillus* spp. and *Fusarium* spp. were identified in cultures from 8 (10%) and 3

(4%) sites, respectively. For the 41 sites with angioinvasion and cultures collected within 24 hours of histopathological specimen, fungal DNA was detected in specimens from 33 sites (81%), with fungi from the order Mucorales being predominant (28 sites; 68%; **Table 11**).

The PCR findings of the full population (not restricted to availability of cultures) were also examined and compared to histopathology (**Tables 12 and 13**). For the 213 injury sites (includes both angioinvasion and nonvascular tissue invasion), fungal DNA was detected in specimens from 60 sites with histopathological evidence of fungal infection, resulting in a sensitivity of 63.2% (**Tables 12 and 14**). Specificity was calculated to be 99.2% based upon the identification of one false-positive detection of *Alternaria* spp. (false positive rate of 0.8%). The PCR-based assay failed to detect fungal DNA in specimens from 35 sites that had histopathological fungal evidence (false negative rate of 36.8%). The positive predictive value of the PCR-based assay was calculated to be 98.4%, while the negative predictive value was 77.0%. Based on the positive and negative likelihood ratios of 75.8 and 0.37, the diagnostic odds ratio is 205, indicating that the PCR assay is better at distinguishing specimens with positive histopathology from negative histopathology.

The PCR findings from sites with positive angioinvasion were also examined (N=166; 48 sites with angioinvasion and 118 sites without; **Table 13**). The sensitivity of the PCR-based assay remained at 99.2% and the negative predictive value increased to 93.6% (fungal DNA detected in specimens from 40 out of 48 sites with angioinvasion). Based on the lack of fungal DNA detection from specimens from eight sites, the false-negative rate decreased to 16.7% (**Table 14**). The diagnostic odds ratio also increased to 578. These findings indicate that PCR is more sensitive than cultures when angioinvasion is present (Kappa coefficient: 0.862; 95% confidence interval: 0.775-0.949).

Specimens from 35 sites with histopathological fungal evidence (8 with angioinvasion and 27 with nonvascular tissue invasion) failed to amplify DNA in the PCR assay. Seven of the 8 sites with angioinvasion had cultures sent for analysis, while it was 23 of the 27 sites with nonvascular tissue invasion with cultures (**Table 15**). Nine sites (four with angioinvasion and five with nonvascular tissue invasion) that had false negative PCR findings grew fungi from the order Mucorales, while six and five sites grew *Aspergillus* spp. and *Fusarium* spp., respectively.

Although they were excluded from the analysis, it is worth noting that common skin and environmental contaminants were detected in specimens from sites with positive histopathology, as well as controls. In particular, *Malassezia* spp. was detected in specimens from 29 sites with positive histopathology. Among these sites, the PCR assay detected fungal DNA from organisms included in the analysis in specimens from 12 sites (17 sites were negative). In addition, *Malassezia* spp. was detected in specimens from 33 control sites. *Candida* spp. (excluded from the analysis) was detected in specimens from three sites with positive histopathology (one site also

positive for filamentous fungi) and two sites from controls. *Aureobisidium pullulans* was detected in specimens with two sites with positive histopathology (one site was positive for filamentous fungi) and three sites from controls. *Yarrowia lipolytica* was only detected in specimens from one control site. *Trichosporon asahii* was detected in specimens from three sites with positive histopathology (all negative for filamentous fungi) and no control sites. *Cladosporium cladosporioides* was detected in specimens from one site with positive histopathology (positive for filamentous fungi) and two control sites.

4.2.5. Concordance of Mycology and PCR-based Assay

In a comparison of findings of PCR and culture data, 11 sites with fungal evidence on histopathology had both negative cultures and negative PCR; however, significant discordance was observed (Kappa coefficient: 0.248; **Table 10**). Nonetheless, PCR was able to identify fungal DNA from the order Mucorales in 43 (49%) injury sites, compared to 31 (36%) with cultures. Between the PCR and culture findings, specimens from 21 injury sites were concordant regarding detection of fungi from the order Mucorales (Kappa coefficient: 0.262), while it was 9 and 3 injury sites for *Aspergillus* spp. and *Fusarium* spp. (Kappa coefficient: 0.387 and 0.208, respectively). When the cultures were restricted to those collected within 24 hours of the histopathological specimen, PCR continued to have a higher detection of fungi from the order Mucorales compared to cultures (48% versus 33%; Kappa coefficient: 0.421) and the number of injury sites with concordant results declined to 20. For *Aspergillus* spp. and *Fusarium* spp., the number of concordant sites declined to 5 and zero, respectively (Kappa coefficient: 0.371 and -0.060, respectively).

When the sites were restricted to those with angioinvasion, both PCR and culture identified fungi from 37 sites with 31 being concordant (**Table 11**). The PCR continued to have a higher detection of fungi from the order Mucorales (75% versus 46%; Kappa coefficient: 0.087) and a lower detection of *Aspergillus* spp. (16% versus 30%; Kappa coefficient: 0.370).

4.2.6. Panfungal vs Semi-nested PCR Assay: Limited Assessment

Sixty-seven blinded specimens (representing 18% of the total 363 specimens) were also sent to the LADR laboratory in Germany for quality assurance, of which 5 were control specimens and 62 were histopathology positive (**Table 16**). The laboratory at UTHSCSA failed to detect fungal DNA in 15 specimens (24% of 62), while fungal DNA was not identified in 6 specimens in LADR (10%). Overall, the results were not concordant (Kappa coefficient: 0.171; 95% confidence interval: -0.09 to 0.432) for identification of fungi at the genus level. Nevertheless, concordance improved with fungi of the order Mucorales (Kappa coefficient: 0.494; 95% confidence interval: 0.297-0.692).

When the specimens were restricted to those with sites with angioinvasion (37 specimens), UTHSCSA and LADR failed to detect fungi in eight and one specimens, respectively (Kappa coefficient: 0.183; 95% confidence interval: -0.131 to 0.497; **Table 17**). Fungi from the order Mucorales were identified in 22 specimens from UTHSCSA (17 with *Saksenaea* spp.), while LADR detected order Mucorales DNA in 31 specimens (13 with *Saksenaea* spp.) resulting in a Kappa coefficient of 0.318 (95% confidence interval: 0.044-0.593) for order Mucorales and 0.446 (95% confidence interval: 0.161-0.731) for *Saksenaea* spp.

Nine specimens from non-trauma comparator subjects were analyzed and the results of the PCR were negative for all but one specimen, which amplified *Malassezia restricta*.

5. DISCUSSION AND RECOMMENDATIONS

To identify eligible subjects for the retrospective analysis, we undertook an evaluation of all subjects with laboratory evidence of fungal infections in the TIDOS population. This report summarizes i) the epidemiologic characteristics of subjects with laboratory evidence of fungal infections in the TIDOS population; ii) refines the previously proposed definition for IFI diagnosis; iii) characterizes subjects into risk groups, namely those with wounds that meet definitions for an IFI, those with wounds that are highly suspicious for an IFI but do not meet criteria for IFI, and wounds which are low suspicion for an IFI; iv) examines the performance characteristics of a PCR-based assay, on FFPE tissue, for the diagnosis of an IFI; and finally v) assesses compliance with the JTS CPG.

5.1. Epidemiology of Combat-related Fungal Wound Infections

During Operation Enduring Freedom, IFI were common and resulted in significant morbidity and mortality.^{2,4} In the comprehensive evaluation herein, 12.7% of all admissions to the participating hospitals in the United States had laboratory evidence of a fungus (either on histopathology and/or cultures), and this proportion was a third among those who had tissue/cultures submitted for examination. Approximately 5% of admissions (13% among those who had tissue for histopathology/cultures submitted) met criteria for an IFI, and ~3% of the subjects had wounds that met criteria for High Suspicion (~8% among those who had tissue for histopathology/ cultures submitted). This represents a significant burden of disease and underscores the need to continue our examinations regarding optimal methods to identify and treat these infections. In general, as previously demonstrated, these individuals were severely injured (approximately 95% sustained dismounted injuries), required massive transfusions, and were commonly admitted to the LRMC intensive care unit. Nevertheless, there were difference between the IFI, High Suspicion, and Low Suspicion groups in terms of their injury severity, proportion of subjects receiving >20 units of red blood cells in a 24-hour interval, LRMC and U.S. hospital SOFA scores, and the need for mechanical ventilation (**Table 5**). While traumatic amputations were common in all three groups, one interesting finding is that there was a trend toward statistical significance with the subjects classified as Low Suspicion (albeit not reaching statistical significance) compared to the IFI

subjects (80% versus 68%; $p=0.060$), which might have reduced the burden of disease in this population. Our results reiterate prior observations that dismounted blast injuries and receipt of blood transfusion as being risk factors for this disease. Nevertheless, only 62% of the subjects classified as either IFI or High Suspicion met the criteria for a DCBI (defined in **Table 6**), indicating that the criteria of complex injury is not adequately sensitive to predict IFI risk.

The distribution of fungi isolated in culture differed between the IFI, High Suspicion, and Low Suspicion groups, with fungi of the order Mucorales predominating among those with IFI, while other fungi (i.e., fungi belonging to genus other than *Aspergillus* and *Fusarium*, or the order Mucorales) were most common in the Low Suspicion group. The distribution of fungi associated with trauma related IFI were different from those observed in transplant recipients where fungi of the genera *Rhizopus* predominate.⁸⁷

Given that the selected subjects from the general combat trauma population were severely injured, baseline demographic, ISS and SOFA scores are unlikely to help in deciding who needs to start on antifungal therapy; however, a group of clinical and microbiologic characteristics may help identify a group of subjects with low risk characteristics in whom antifungals can be discontinued with close clinical follow-up. The presence of ongoing necrosis, wounds that meet Centers for Disease Control and Prevention National Healthcare Safety Network criteria for skin and soft-tissue infections attributable to the fungus,⁸⁸ and isolation of fungi of the order Mucorales, genus *Aspergillus*, or the genus *Fusarium* identify a group of individuals in whom antifungals should be continued. Furthermore, the high proportion of fungi from the order Mucorales supports use of liposomal Amphotericin B when invasive filamentous fungi are present.

Another takeaway is the high injury severity of the subjects without histopathological evidence of a fungal or bacterial infection (control population). This comparator population was selected based on the availability of archived tissue specimens that had been submitted for histopathology. Therefore, it follows that these patients had serious injuries, prompting concern for IFI. Although there were statistically significant differences between the control and positive histopathology groups, it is important to note that 80% of the control subjects were injury via a blast mechanism (majority dismounted) with 50% having critical injury severity (ISS ≥ 26). In addition, 21% sustained above knee amputations and 62% had perineal, genitourinary, or abdominal injuries. The control subjects did have a lower proportion of large-volume blood transfusions (>20 units) compared to the positive histopathology subjects (20% vs 84%). While the reasons for why subjects with similar injury patterns did not develop disease is unclear, it is possible that the subjects with positive histopathology experienced transient immunosuppression from the receipt of massive blood transfusions, making them more susceptible to infection.⁸⁹⁻⁹¹ Furthermore, the blood transfusions may have resulted in a state of iron overload, which has been associated with increased risk of invasive mucormycosis.^{92,93} While these are potential hypotheses, the need for 20 or more units of packed red blood cells in a 24-hour period is more likely a marker of the

severity of injury (which is not always captured well with the ISS scores) with the resultant ischemia and tissue necrosis, which predisposes to fungal infection. Host and fungal factors that predispose to infection should be further studied.

5.2. PCR Identification of Fungi from Formalin-Fixed Paraffin-Embedded Tissue

The results of our study suggest that it is possible to amplify fungus from FFPE specimens with a high degree of specificity, albeit, with some reduced sensitivity. When compared with histopathology, the sensitivity and specificity of our PCR-based assay was 63% and 99%, respectively, which is comparable to other PCR-based assays (**Table 4**). Higher detection was evident with specimens from sites with angioinvasion (sensitivity: 83%). The timing of tissue specimen collection may have impacted the amplification of fungal DNA using the PCR-based assay. All of the specimens for the PCR analysis were collected following admission to the U.S. hospitals and there is the potential that subjects with nonvascular tissue invasion did not have significant fungal burden reducing the yield on PCR. Nevertheless, the use of specimens from LRMC could have resulted in the identification of fungi that were colonizing the wound rather than being the true pathogen, hence we believe that our rationale of using specimens collected in the U.S. is a reasonable strategy for this analysis. Age of the specimen may have also impacted the results of the PCR-based assay as many were collected seven years prior to the analysis. Historically, FFPE specimens have been challenging for PCR and sequencing-based diagnostics due to DNA degradation and contamination. Often there are multiple technicians in the sample workflow including surgeons, pathologists, technicians who handle and cut paraffin blocks, and the end user (microbiologist/molecular biologist), which increases the risk of contamination. The cutting and extraction method in this analysis were standardized and automated, keeping contamination to a minimum thereby contributing to the high specificity observed in this study. Of the specimens from 118 control sites submitted for analysis, the PCR identified a possible pathogen (*Alternaria* spp.) from one site (0.8%). In addition, skin and environmental contaminants were identified from both subjects with positive histopathology and controls (Ex., *Malassezia* spp. identified in 30% of positive histopathology sites and 28% of control sites).

In prior analyses, fungi from the order Mucorales have been indicated to be the primary pathogenic agents in relation to soft-tissue injuries with soil contamination which is consistent with our results.^{10,11,15,94} Our findings showed that cultures had a lower rate of detection of fungi of the order Mucorales compared to PCR. Even after restricting to specimens collected from sites with angioinvasion, the proportion of cultures where fungi from the order Mucorales were isolated was lower than that identified by PCR. On the other hand, PCR from the primary lab at UTHSCSA was less likely to identify fungi of the genus *Aspergillus* and *Fusarium*. In over half of the cases with documented angioinvasion, fungi of the genera *Saksenaea* were identified, and in combination with fungi of the genus *Apophysomyces* they accounted for two-thirds of the cases with identifiable fungi. These findings are in contrast to the clinical mycology of mucormycosis

diagnosed in immunocompromised patients, such as those undergoing transplants. In particular, the most common fungi among hematopoietic stem cell transplant patients are *Rhizopus* spp. (53%), *Mucor* spp. (16%), *Rhizomucor* spp. (6%), and *Lichtheimia* spp. (6%).⁸⁷ It is recognized that culture is insensitive for identifying fungi from the order Mucorales. Tissue prepared for culture is often ground up and the grinding procedure results in destruction of the hyphal elements, with the resultant reduction in growth.^{95,96} Further, the current culture methods fail to mimic physiologic conditions that are required for fungal growth, thereby decreasing yield.

Nevertheless, it is unclear why the primary lab failed to identify fungi of the genus *Aspergillus* and *Fusarium* when there was growth in culture and specimens from the secondary lab (LADR) identified the organism. One potential explanation is differences in the extraction methods with the secondary lab requiring an additional boiling steps to antagonize the protein complexing effect of formalin. Boiling results in the release of proteins and the amount of protein found in the supernatant correlate with the duration of boiling.⁹⁷ Since histones around the DNA are complexed by formalin, the boiling step should increase the amount of DNA released for amplification. Second, the PCR technique employed by the secondary lab was a semi-nested assay versus a single round PCR method used by the primary lab. Lastly, perhaps the biggest difference between the two techniques is the number of nucleotides required for an identification with the secondary lab requiring a lower number of nucleotides to determine a match. Targeting a more specific sequence in contrast to a broad-spectrum PCR means that the primers detect and bind only to their specific sequences whereas broad-spectrum primers will detect and bind to DNA of all fungi. While the use of lower number of nucleotides to call a match improves the sensitivity of the PCR-based method, it does reduce the specificity of the technique.

The trade-off between specificity and sensitivity is one worth considering. The decision to initiate antifungals is based on easily ascertainable clinical criteria (specified in the JTS CPG)^{7,36} and not the result of laboratory criteria. The identification of fungi of the order Mucorales in a large number of these specimens suggests that the use of broad-spectrum antifungals which cover this order should remain our strategy. Hence, it is unlikely that the failure to identify fungi from the genus *Aspergillus* would alter our clinical strategy. Nonetheless, we need to continue to work on strategies that will improve sensitivity, without reducing specificity. While not tested in this study specifically, we believe the use of fresh tissue for PCR may alleviate these concerns.

Although the PCR sensitivity was lower than the culture findings (63% versus 78%), we believe that the PCR-based assay is valuable. For one, the significant proportion of fungi of the order Mucorales isolated on PCR in comparison to cultures; reliance on cultures alone may have resulted in unfavorable outcomes. Accurate identification of the fungal organism is essential for selection of appropriate antifungal therapy. Further, the results of PCR suggest that our strategy to use early broad-spectrum antifungal coverage with both polyene compounds (Amphotericin-based compounds) and the azole voriconazole is justified.

Other potential benefits of this PCR-based assay include a quick turnaround and species-level identification. In the era of growing antifungal resistance, species level identification may provide insight into the potential for resistance.⁹⁸ Regarding limitations, there is the potential for occurrence of false negatives (37%); however, the proportion of false negative did decrease with specimens from sites with angioinvasion (17%). In addition, moving to a PCR only method would mean isolates would be unavailable for antifungal susceptibility testing if required. Nonetheless, we believe that the pros outweigh the cons regarding the utility of the assay. As a result, we believe that the PCR-based assay should be used as a complement to histopathology/culture to support the diagnosis of IFIs and direct antifungal treatment when the finding is positive.

5.3. Limitations

There are limitations of this analysis that should be taken into consideration. First, the selection of the study population based on histopathological evidence could have biased results relating to the predictive value of cultures. Second, the PCR was conducted using FFPE archived tissue specimens rather than fresh tissue. As previously stated, the sensitivity of the primary PCR-based method was lower for fungi belonging to the genera *Aspergillus* and *Fusarium*.

Furthermore, in this study, we used a standard primer pair and limited the PCR reactions to a single run. In many cases, multiple reactions were performed, and different primer combinations were sometimes used. This strategy would only be feasible in an experienced molecular laboratory.

5.4. Adherence to DoD Recommendations

To be considered at risk for an IFI by the 2012 JTS CPG, a subject was required to meet at least three risk factors: 1) dismounted blast injury; 2) above knee traumatic amputation (or progressive transition from below knee to through knee to above knee amputation); 3) extensive perineal, genitourinary, and/or rectal injury; and 4) massive transfusion >20 units packed red blood cells plus whole blood (the CPG listed >25 units; however, further examination of risk factors identified >20 units as an independent predictor).^{7,8} Among the 131 IFI and High Suspicion of IFI subjects in our population (24 subjects with missing mounted status excluded), 114 (87%) met the JTS criteria for at risk for IFI. Our findings support and strengthen the recommendations in the JTS CPG regarding collection of specimens from patients at risk for IFIs. As dissemination of recommendations is only one part of the equation, adherence to the CPG was examined.

Per the 2012 JTS CPG related to the management of IFIs in war wounds,⁷ the collections of tissue specimens at Role 4 facilities (LRMC) from patients meeting at least three risk factors for IFI was recommended. Following admission in the United States (Role 5), it was recommended to also collect tissue specimens from patients with at least three IFI risk factors as well as a suspicious wound (e.g., tissue necrosis).

Among 1900 combat casualties from OEF with open wounds admitted following the implementation of the LRMC Blast Protocol (February 2011 - December 2014), 198 (10%) met the criteria for being at risk for IFI (**Table 2**) and 140 (7%) had a DCBI (**Table 18**). Based on the JTS recommendations and the LRMC Blast Protocol, all of these patients should have had specimens collected for culture and histopathological analysis. Tissue specimens for histopathology were collected from 144 (73%) of the patients at risk for IFI at LRMC, while cultures were obtained from 145 (73%) patients. Among the 180 who were admitted to participating U.S. hospitals, 124 (69%) and 143 (79%) had specimens collected for histopathology and culture, respectively. Furthermore, 104 (74%) of the 140 DCBI patients at LRMC had specimens collected for both histopathology and 105 (75%) had cultures collected. Following admission of 130 DCBI patients to U.S. hospitals, 90 (69% of 130) and 103 (79%) patients had specimens collected for histopathology and cultures, respectively.

Examination of the period prior to the implementation of the LRMC Blast Protocol (June 2009 – January 2011) determined a much lower proportion of specimens collected from subjects with blast trauma. A total of 93 patients were classified as at risk for IFI at LRMC, of which 85 were admitted to participating U.S. hospitals. Specimens were collected for histopathology from 12 (13%) and 30 (35%) of the patients at LRMC and U.S. hospitals, respectively (26 [28%] and 46 (54%) patients with cultures collected, respectively). For the 53 patients who met the conditions of a DCBI (46 admitted to participating U.S. hospitals), 10 (19%) and 19 (41%) patients had tissue specimens collected for histopathology at LRMC and the U.S. hospitals, respectively, while 17 (32%) and 27 (59%) patients had cultures collected, respectively.

5.5. Recommendations

The 2011 TIDOS IFI Case Investigation Report determined that the critical elements for management of IFIs includes key awareness of risk factors and common clinical findings, early diagnosis, appropriate handling of clinical specimens, aggressive surgical debridement, and use of empiric antifungal therapy where there is a strong clinical suspicion of an IFI. The findings of this report herein strengthens these recommendations and indicate that the PCR-based assay is more effective at detecting pathogenic fungi (particularly *Saksena* spp.) when compared to conventional cultures, providing increased accuracy and timeliness to the diagnostic test strategy. Our recommendations are as follows:

1. PCR-based assay should be utilized as a complement to histopathology and cultures to direct antifungal treatment
2. Specimens for PCR analysis should be collected following the completion of at least two surgical debridements after evacuation from the operational theater in order to avoid contamination and false-positive results
3. Initial coverage to include dual antifungals (i.e., liposomal Amphotericin B and voriconazole/posaconazole) should be directed toward the order Mucorales in patients with histopathological evidence of invasive filamentous fungi

4. Based on discordant findings between the panfungal and semi-nested PCRs, the relative performance of the two PCR based methods, including testing of both traditional and real-time PCR on both fresh and FFPE, should be the focus of future work

5.6. Follow-on Work

Based on the findings of this investigation and input from subject-matter expert panel review, we propose additional analyses are warranted. These include:

Follow-on PCR-based investigation to inform DoD diagnostic platforms

- a) Assess test performance of semi-nested PCR in comparison to panfungal PCR-based method
- b) Improving the sensitivity of the PCR-based methods of FFPE specimens through examination of probes
- c) Examination of technical issues related to specimen processing (e.g., boiling step) that could lead to an improved yield and improved sensitivity in samples with non-vascular tissue invasion
- d) Expand PCR analysis to include patients classified as Possible IFI and Low Suspicion who had negative histopathology findings to assess test performance
- e) Targeted analysis of PCR testing on serial samples to evaluate differences across timeframe of disease (early at LRMC to later specimens as IFI under directed antifungal/surgical care)

Apply PCR-based etiology to further understanding of patient risk, co-infections, and clinical outcomes

- a) Examination of clinical outcomes utilized the refined classifications IFI, High Suspicion, and Low Suspicion, as well as the updated IFI Case Registry
- b) Comparison of clinical outcomes among IFI patients with *Saksenaea* spp.
- c) Refinement of a clinical decision support tool to assist with IFI diagnosis

Other considerations include the evaluation of the performance characteristics of the PCR-based method on fresh tissue specimens if there is another conflict. In addition, the suitability of an animal model can also be assessed, as well as a clinical trial to examine whether outcomes are improved when a PCR-based method is used as a complement to ongoing laboratory methods, optimal debridement, and antifungal therapy.

6. ACKNOWLEDGMENTS

We would like to thank the Infectious Disease Clinical Research Program of the Uniformed Services University of the Health Sciences, Trauma Infectious Disease Outcomes Study team of clinical coordinators, microbiology technicians, data managers, clinical site managers, and administrative support personnel for their hard work to ensure the success of this project. This work would also not have been possible without the support of our team at Walter Reed National Military Medical Center, Brooke Army Medical Center, and Landstuhl Regional Medical Center for their work and support. We also wish to specifically thank our collaborators of Dr. Brian

Wickes and his team at the University of Texas Health Sciences Center San Antonio, Texas, as well as Dr. Ralf Bialek and his team at LADR GmbH MVZ Dr. Kramer & Kollegen, Germany for their hard work. Lastly, we wish to thank the Defense Medical Research and Development Program for funding this project.

7. REFERENCES

1. Ficke JR, Eastridge BJ, Butler F, Alvarez J, Brown T, Pasquina P, Stoneman P, Carvalho JJ. Dismounted complex blast injury report of the army dismounted complex blast injury task force. *J Trauma Acute Care Surg*. 2012;73(6 Suppl 5):S520-S534.
2. Warkentien T, Rodriguez C, Lloyd B, Wells J, Weintrob A, Dunne J, Ganesan A, Li P, Bradley W, Gaskins LJ, et al. Invasive mold infections following combat-related injuries. *Clin Infect Dis*. 2012;55(11):1441-1449.
3. Evriviades D, Jeffery S, Cubison T, Lawton G, Gill M, Mortiboy D. Shaping the military wound: issues surrounding the reconstruction of injured servicemen at the Royal Centre for Defence Medicine. *Philos Trans R Soc Lond B Biol Sci*. 2011;366(1562):219-230.
4. Weintrob AC, Weisbrod AB, Dunne JR, Rodriguez CJ, Malone D, Lloyd BA, Warkentien T, Wells J, Murray CK, Bradley W, et al. Combat Trauma-Associated Invasive Fungal Wound Infections: Epidemiology and Clinical Classification. *Epidemiol Infect*. 2015;143(1):214-224.
5. Tribble DR, Conger NG, Fraser S, Gleeson TD, Wilkins K, Antonille T, Weintrob A, Ganesan A, Gaskins LJ, Li P, et al. Infection-associated clinical outcomes in hospitalized medical evacuees after traumatic injury: trauma infectious disease outcome study. *J Trauma*. 2011;71(1 Suppl):S33-S42.
6. Trauma Infectious Diseases Outcomes Study Group. Department of Defense Technical Report - Invasive Fungal Infection Case Investigation. Bethesda, MD: Infectious Disease Clinical Research Program, Uniformed Services University of the Health Sciences; April 11, 2011.
7. Joint Theater Trauma System. Treatment of suspected invasive fungal infection in war wounds. 2012; Available from: http://www.usaisr.amedd.army.mil/assets/cpgs/Invasive_Fungal_Infection_in_War_Wounds_1_Nov_12.pdf.
8. Rodriguez C, Weintrob AC, Shah J, Malone D, Dunne JR, Weisbrod AB, Lloyd BA, Warkentien T, Murray CK, Wilkins K, et al. Risk factors associated with invasive fungal Infections in combat trauma. *Surg Infect (Larchmt)*. 2014;15(5):521-526.
9. Lewandowski LR, Weintrob AC, Tribble DR, Rodriguez CJ, Petfield J, Lloyd BA, Murray CK, Stinner D, Aggarwal D, Shaikh F, et al. Early Complications and Outcomes in Combat Injury Related Invasive Fungal Wound Infections: A Case-Control Analysis. *J Orthop Trauma*. 2016;30(3):e93-e99.
10. Warkentien TE, Shaikh F, Weintrob AC, Rodriguez CJ, Murray CK, Lloyd BA, Ganesan A, Aggarwal D, Carson ML, Tribble DR, et al. Impact of Mucorales and Other Invasive Molds on Clinical Outcomes of Polymicrobial Traumatic Wound Infections. *J Clin Microbiol*. 2015;53(7):2262-2270.
11. Kronen R, Liang SY, Bochicchio G, Bochicchio K, Powderly WG, Spec A. Invasive Fungal Infections Secondary to Traumatic Injury. *Int J Infect Dis*. 2017;62:102-111.

12. Vitrat-Hincky V, Lebeau B, Bozonnet E, Falcon D, Pradel P, Faure O, Aubert A, Piolat C, Grillot R, Pelloux H. Severe filamentous fungal infections after widespread tissue damage due to traumatic injury: six cases and review of the literature. *Scand J Infect Dis*. 2009;41(6-7):491-500.
13. Lelievre L, Garcia-Hermoso D, Abdoul H, Hivelin M, Chouaki T, Toubas D, Mamez AC, Lantieri L, Lortholary O, Lanternier F, et al. Posttraumatic mucormycosis: a nationwide study in France and review of the literature. *Medicine (Baltimore)*. 2014;93(24):395-404.
14. Jain D, Kumar Y, Vasishta RK, Rajesh L, Pattari SK, Chakrabarti A. Zygomycotic necrotizing fasciitis in immunocompetent patients: a series of 18 cases. *Mod Pathol*. 2006;19(9):1221-1226.
15. Lanternier F, Dannaoui E, Morizot G, Elie C, Garcia-Hermoso D, Huerre M, Bitar D, Dromer F, Lortholary O, French Mycosis Study G. A global analysis of mucormycosis in France: the RetroZygo Study (2005-2007). *Clin Infect Dis*. 2012;54 Suppl 1:S35-43.
16. Kennedy KJ, Daveson K, Slavin MA, van Hal SJ, Sorrell TC, Lee A, Marriott DJ, Chapman B, Halliday CL, Hajkiewicz K, et al. Mucormycosis in Australia: contemporary epidemiology and outcomes. *Clin Microbiol Infect*. 2016.
17. Chander J, Singla N, Kaur M, Punia RS, Attri A, Alastruey-Izquierdo A, Cano-Lira JF, Stchigel AM, Guarro J. *Saksena* erythrospora, an emerging mucoralean fungus causing severe necrotizing skin and soft tissue infections - a study from a tertiary care hospital in north India. *Infect Dis (Lond)*. 2017;49(3):170-177.
18. Patino JF, Castro D, Valencia A, Morales P. Necrotizing soft tissue lesions after a volcanic cataclysm. *World J Surg*. 1991;15(2):240-247.
19. Cocanour CS, Miller-Crotchett P, Reed RL, 2nd, Johnson PC, Fischer RP. Mucormycosis in trauma patients. *J Trauma*. 1992;32(1):12-15.
20. Johnson PC, Satterwhite TK, Monheit JE, Parks D. Primary cutaneous mucormycosis in trauma patients. *J Trauma*. 1987;27(4):437-441.
21. Gordon G, Indeck M, Bross J, Kapoor DA, Brotman S. Injury from silage wagon accident complicated by mucormycosis. *J Trauma*. 1988;28(6):866-867.
22. Roden MM, Zaoutis TE, Buchanan WL, Knudsen TA, Sarkisova TA, Schaufele RL, Sein M, Sein T, Chiou CC, Chu JH, et al. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. *Clin Infect Dis*. 2005;41(5):634-653.
23. Andresen D, Donaldson A, Choo L, Knox A, Klaassen M, Ursic C, Vonthehoff L, Krilis S, Konecny P. Multifocal cutaneous mucormycosis complicating polymicrobial wound infections in a tsunami survivor from Sri Lanka. *Lancet*. 2005;365(9462):876-878.
24. Moran SL, Strickland J, Shin AY. Upper-extremity mucormycosis infections in immunocompetent patients. *J Hand Surg Am*. 2006;31(7):1201-1205.
25. Neblett Fanfair R, Benedict K, Bos J, Bennett SD, Lo Y-C, Adebajo T, Etienne K, Deak E, Derado G, Shieh W-J, et al. Necrotizing Cutaneous Mucormycosis after a Tornado in Joplin, Missouri, in 2011. *N Engl J Med*. 2012;367(23):2214-2225.
26. Snell BJ, Tavakoli K. Necrotizing fasciitis caused by *Apophysomyces* elegans complicating soft-tissue and pelvic injuries in a tsunami survivor from Thailand. *Plast Reconstr Surg*. 2007;119(1):448-449.
27. Van Sickels N, Hoffman J, Stuke L, Kempe K. Survival of a patient with trauma-induced mucormycosis using an aggressive surgical and medical approach. *J Trauma*. 2011;70(2):507-509.
28. Gomez-Camarasa C, Rojo-Martin MD, Miranda-Casas C, Alastruey-Izquierdo A, Aliaga-Martinez L, Labrador-Molina JM, Navarro-Mari JM. Disseminated Infection due to *Saksena* vasiformis Secondary to Cutaneous Mucormycosis. *Mycopathologia*. 2013.

29. Ingram PR, Suthanathan AE, Rajan R, Pryce TM, Sieunarine K, Gardam DJ, Heath CH. Cutaneous mucormycosis and motor vehicle accidents: Findings from an Australian case series. *Med Mycol*. 2014.
30. Coronel-Perez IM, Rodriguez-Rey EM, Castilla-Guerra L, Dominguez MC. Primary Cutaneous Mucormycosis Due to *Saksenaea vasiformis* in an Immunocompetent Patient. *Actas Dermosifiliogr*. 2015.
31. Lloyd B, Weintrob A, Rodriguez C, Dunne J, Weisbrod A, Hinkle M, Warkentien T, Murray C, Oh J, Millar E, et al. Effect of early screening for invasive fungal infections in U.S. service members with explosive blast injuries. *Surg Infect (Larchmt)*. 2014;15(5):619-626.
32. Barsoumian A, Sanchez CJ, Mende K, Tully CC, Beckius ML, Akers KS, Wenke JC, Murray CK. In Vitro Toxicity and Activity of Dakin's Solution, Mafenide Acetate, and Amphotericin B on Filamentous Fungi and Human Cells. *J Orthop Trauma*. 2013;27(8):428-436.
33. Lewandowski L, Purcell R, Fleming M, Gordon WT. The Use of Dilute Dakin's Solution for the Treatment of Angioinvasive Fungal Infection in the Combat Wounded: A Case Series. *Mil Med*. 2013;178(4):e503-e507.
34. Blyth DM, Chung KK, Cancio LC, King BT, Murray CK. Clinical utility of fungal screening assays in adults with severe burns. *Burns*. 2013;39(3):413-419.
35. Heaton SM, Weintrob AC, Downing K, Keenan B, Aggarwal D, Shaikh F, Tribble DR, Wells J. Histopathological techniques for the diagnosis of combat-related invasive fungal wound infections. *BMC Clinical Pathology*. 2016;16(1):1-9.
36. Rodriguez CJ, Tribble DR, Murray CK, Jessie EM, Fleming ME, Potter BK, Gordon WT, Shackelford SA. Invasive fungal infection in war wounds (CPG: 28). Joint Trauma System; 2016.
37. Tarrand JJ, Lichterfeld M, Warraich I, Luna M, Han XY, May GS, Kontoyiannis DP. Diagnosis of invasive septate mold infections. A correlation of microbiological culture and histologic or cytologic examination. *Am J Clin Pathol*. 2003;119(6):854-858.
38. Schofield CM, Murray CK, Horvath EE, Cancio LC, Kim SH, Wolf SE, Hospenthal DR. Correlation of culture with histopathology in fungal burn wound colonization and infection. *Burns*. 2007;33(3):341-346.
39. Rickerts V, Mousset S, Lambrecht E, Tintelnot K, Schwerdtfeger R, Presterl E, Jacobi V, Just-Nubling G, Bialek R. Comparison of histopathological analysis, culture, and polymerase chain reaction assays to detect invasive mold infections from biopsy specimens. *Clin Infect Dis*. 2007;44(8):1078-1083.
40. Becker WK, Cioffi WG, Jr., McManus AT, Kim SH, McManus WF, Mason AD, Pruitt BA, Jr. Fungal burn wound infection. A 10-year experience. *Arch Surg*. 1991;126(1):44-48.
41. Tarrand JJ, Han XY, Kontoyiannis DP, May GS. *Aspergillus* hyphae in infected tissue: evidence of physiologic adaptation and effect on culture recovery. *J Clin Microbiol*. 2005;43(1):382-386.
42. Boucher HW, Groll AH, Chiou CC, Walsh TJ. Newer systemic antifungal agents : pharmacokinetics, safety and efficacy. *Drugs*. 2004;64(18):1997-2020.
43. Bellmann R, Cleary JD, Sterba J. Clinical roundtable monograph: safety and efficacy of lipid-based amphotericin B. *Clin Adv Hematol Oncol*. 2009;7(4):1-8.
44. Bruggemann RJ, Alffenaar JW, Blijlevens NM, Billaud EM, Kosterink JG, Verweij PE, Burger DM. Clinical relevance of the pharmacokinetic interactions of azole antifungal drugs with other coadministered agents. *Clin Infect Dis*. 2009;48(10):1441-1458.

45. Visser DH, van den Berg YL, van Furth AM, Oomen MW, Schouten-van Meeteren AY, Pajkrt D, van den Bos C, van Well GT. Diagnosis and treatment of cutaneous zygomycosis. *Pediatr Infect Dis J*. 2007;26(12):1165-1166.
46. Chamilos G, Lewis RE, Kontoyiannis DP. Delaying amphotericin B-based frontline therapy significantly increases mortality among patients with hematologic malignancy who have zygomycosis. *Clin Infect Dis*. 2008;47(4):503-509.
47. Yang M, Lee JH, Kim YK, Ki CS, Huh HJ, Lee NY. Identification of mucorales from clinical specimens: a 4-year experience in a single institution. *Ann Lab Med*. 2016;36(1):60-63.
48. Cuenca-Estrella M, Bassetti M, Lass-Flörl C, Racil Z, Richardson M, Rogers TR. Detection and investigation of invasive mould disease. *J Antimicrob Chemother*. 2011;66 Suppl 1:i15-24.
49. Chen SC, Kontoyiannis DP. New molecular and surrogate biomarker-based tests in the diagnosis of bacterial and fungal infection in febrile neutropenic patients. *Curr Opin Infect Dis*. 2010;23(6):567-577.
50. Springer J, Goldenberger D, Schmidt F, Weisser M, Wehrle-Wieland E, Einsele H, Frei R, Löffler J. Development and application of two independent real-time PCR assays to detect clinically relevant Mucorales species. *J Med Microbiol*. 2016;65(3):227-234.
51. Springer J, Lackner M, Ensinger C, Risslegger B, Morton CO, Nachbaur D, Lass-Flörl C, Einsele H, Heinz WJ, Loeffler J. Clinical Evaluation of Mucorales-specific Real-time PCR Assay in Tissue and Serum Samples. *J Med Microbiol*. 2016;65(12):1414-1421.
52. Drogari-Apiranthitou M, Panayiotides I, Galani I, Konstantoudakis S, Arvanitidis G, Spathis A, Gouloumi AR, Tsakiraki Z, Tsiodras S, Petrikos G. Diagnostic value of a semi-nested PCR for the diagnosis of mucormycosis and aspergillosis from paraffin-embedded tissue: A single center experience. *Pathol Res Pract*. 2016;212(5):393-397.
53. Millon L, Herbrecht R, Grenouillet F, Morio F, Alanio A, Letscher-Bru V, Cassaing S, Chouaki T, Kauffmann-Lacroix C, Poirier P, et al. Early diagnosis and monitoring of mucormycosis by detection of circulating DNA in serum: retrospective analysis of 44 cases collected through the French Surveillance Network of Invasive Fungal Infections (RESSIF). *Clin Microbiol Infect*. 2016;22(9):810.e811-e818.
54. Millon L, Larosa F, Lepiller Q, Legrand F, Rocchi S, Daguindau E, Scherer E, Bellanger AP, Leroy J, Grenouillet F. Quantitative polymerase chain reaction detection of circulating DNA in serum for early diagnosis of mucormycosis in immunocompromised patients. *Clin Infect Dis*. 2013;56(10):e95-101.
55. Alanio A, Garcia-Hermoso D, Mercier-Delarue S, Lanternier F, Gits-Muselli M, Menotti J, Denis B, Bergeron A, Legrand M, Lortholary O, et al. Molecular identification of Mucorales in human tissues: contribution of PCR electrospray-ionization mass spectrometry. *Clin Microbiol Infect*. 2015.
56. Shigemura T, Nakazawa Y, Matsuda K, Sano K, Yaguchi T, Motobayashi M, Saito S, Noda S, Kobayashi N, Agematsu K, et al. Serial monitoring of Mucorales DNA load in serum samples of a patient with disseminated mucormycosis after allogeneic bone marrow transplantation. *Int J Hematol*. 2014;100(2):206-209.
57. Hebart H, Löffler J, Meisner C, Serey F, Schmidt D, Bohme A, Martin H, Engel A, Bunje D, Kern WV, et al. Early detection of aspergillus infection after allogeneic stem cell transplantation by polymerase chain reaction screening. *J Infect Dis*. 2000;181(5):1713-1719.
58. Hebart H, Löffler J, Reitze H, Engel A, Schumacher U, Klingebiel T, Bader P, Bohme A, Martin H, Bunjes D, et al. Prospective screening by a panfungal polymerase chain reaction assay

in patients at risk for fungal infections: implications for the management of febrile neutropenia. *Br J Haematol.* 2000;111(2):635-640.

59. Williamson EC, Leeming JP, Palmer HM, Steward CG, Warnock D, Marks DI, Millar MR. Diagnosis of invasive aspergillosis in bone marrow transplant recipients by polymerase chain reaction. *Br J Haematol.* 2000;108(1):132-139.

60. Raad I, Hanna H, Huaranga A, Sumoza D, Hachem R, Albitar M. Diagnosis of invasive pulmonary aspergillosis using polymerase chain reaction-based detection of aspergillus in BAL. *Chest.* 2002;121(4):1171-1176.

61. Raad I, Hanna H, Sumoza D, Albitar M. Polymerase chain reaction on blood for the diagnosis of invasive pulmonary aspergillosis in cancer patients. *Cancer.* 2002;94(4):1032-1036.

62. Buchheidt D, Baust C, Skladny H, Ritter J, Suedhoff T, Baldus M, Seifarth W, Leib-Moesch C, Hehlmann R. Detection of *Aspergillus* species in blood and bronchoalveolar lavage samples from immunocompromised patients by means of 2-step polymerase chain reaction: clinical results. *Clin Infect Dis.* 2001;33(4):428-435.

63. Halliday C, Hoile R, Sorrell T, James G, Yadav S, Shaw P, Bleakley M, Bradstock K, Chen S. Role of prospective screening of blood for invasive aspergillosis by polymerase chain reaction in febrile neutropenic recipients of haematopoietic stem cell transplants and patients with acute leukaemia. *Br J Haematol.* 2006;132(4):478-486.

64. Mengoli C, Cruciani M, Barnes RA, Loeffler J, Donnelly JP. Use of PCR for diagnosis of invasive aspergillosis: systematic review and meta-analysis. *Lancet Infect Dis.* 2009;9(2):89-96.

65. Badiie P, Arastefar A, Jafarian H. Comparison of histopathological analysis, culture and polymerase chain reaction assays to detect mucormycosis in biopsy and blood specimens. *Iran J Microbiol.* 2013;5(4):406-410.

66. Bernal-Martinez L, Buitrago MJ, Castelli MV, Rodriguez-Tudela JL, Cuenca-Estrella M. Development of a single tube multiplex real-time PCR to detect the most clinically relevant *Mucormycetes* species. *Clin Microbiol Infect.* 2013;19(1):E1-7.

67. Bialek R, Konrad F, Kern J, Aepinus C, Cecenas L, Gonzalez GM, Just-Nubling G, Willinger B, Presterl E, Lass-Flörl C, et al. PCR based identification and discrimination of agents of mucormycosis and aspergillosis in paraffin wax embedded tissue. *J Clin Pathol.* 2005;58(11):1180-1184.

68. Babouee B, Goldenberger D, Elzi L, Lardinois D, Sadowski-Cron C, Bubendorf L, Savic Prince S, Battegay M, Frei R, Weisser M. Prospective study of a panfungal PCR assay followed by sequencing, for the detection of fungal DNA in normally sterile specimens in a clinical setting: a complementary tool in the diagnosis of invasive fungal disease? *Clin Microbiol Infect.* 2013;19(8):E354-357.

69. Salehi E, Hedayati MT, Zoll J, Rafati H, Ghasemi M, Doroudinia A, Abastabar M, Tolooe A, Snelders E, van der Lee HA, et al. Discrimination of aspergillosis, mucormycosis, fusariosis and scedosporiosis in formalin-fixed paraffin-embedded tissue specimens using multiple real-time quantitative PCR assays. *J Clin Microbiol.* 2016;54(11):2798-2803.

70. Lau A, Chen S, Sorrell T, Carter D, Malik R, Martin P, Halliday C. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. *J Clin Microbiol.* 2007;45(2):380-385.

71. Munoz-Cadavid C, Rudd S, Zaki SR, Patel M, Moser SA, Brandt ME, Gomez BL. Improving molecular detection of fungal DNA in formalin-fixed paraffin-embedded tissues: comparison of five tissue DNA extraction methods using panfungal PCR. *J Clin Microbiol.* 2010;48(6):2147-2153.

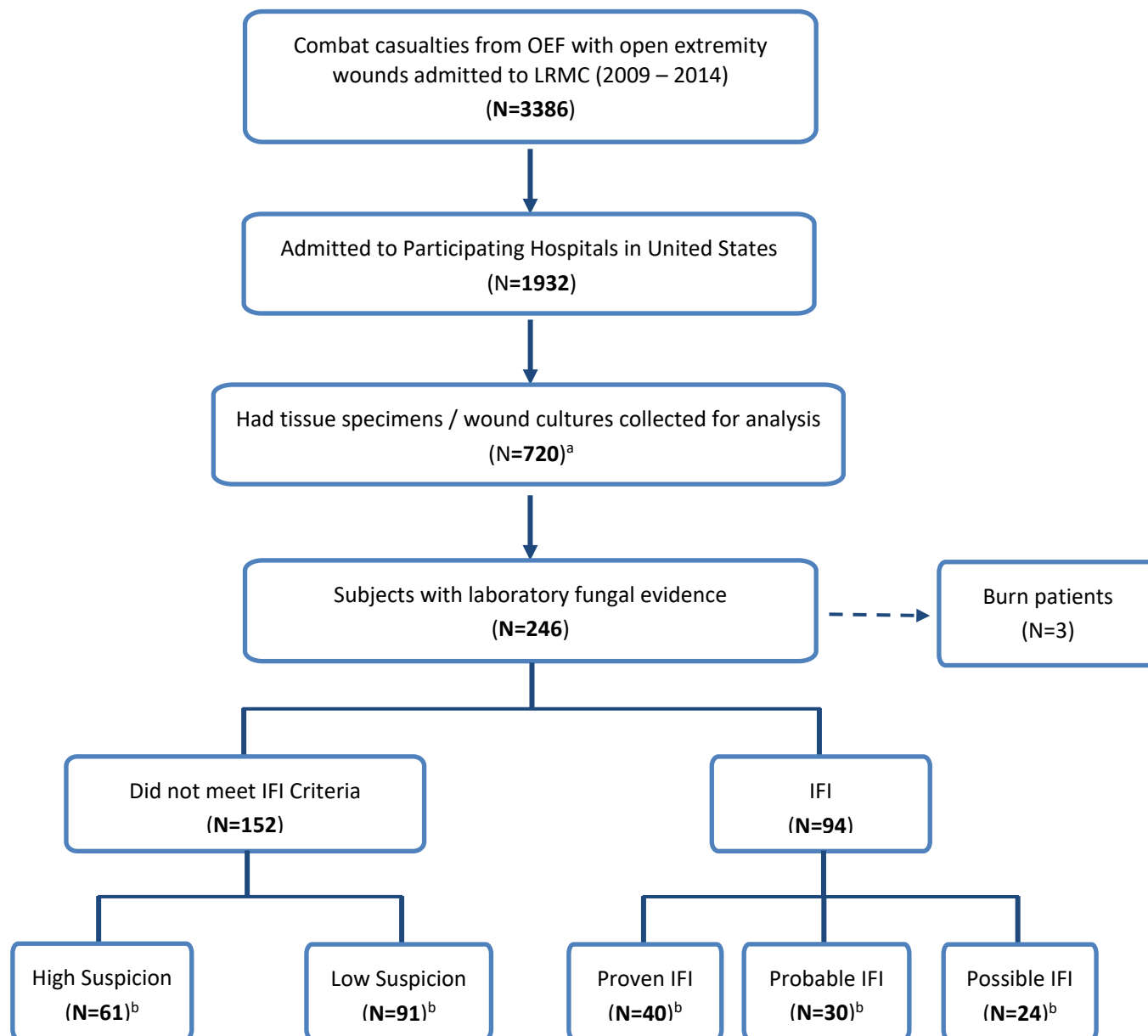
72. Hata DJ, Buckwalter SP, Pritt BS, Roberts GD, Wengenack NL. Real-time PCR method for detection of zygomycetes. *J Clin Microbiol*. 2008;46(7):2353-2358.
73. Buitrago MJ, Aguado JM, Ballen A, Bernal-Martinez L, Prieto M, Garcia-Reyne A, Garcia-Rodriguez J, Rodriguez-Tudela JL, Cuenca-Estrella M. Efficacy of DNA amplification in tissue biopsy samples to improve the detection of invasive fungal disease. *Clin Microbiol Infect*. 2013;19(6):E271-277.
74. Moncada PA, Budvytiene I, Ho DY, Deresinski SC, Montoya JG, Banaei N. Utility of DNA sequencing for direct identification of invasive fungi from fresh and formalin-fixed specimens. *Am J Clin Pathol*. 2013;140(2):203-208.
75. Rickerts V. Identification of fungal pathogens in Formalin-fixed, Paraffin-embedded tissue samples by molecular methods. *Fungal Biol*. 2016;120(2):279-287.
76. Babouee Flury B, Weisser M, Prince SS, Bubendorf L, Battegay M, Frei R, Goldenberger D. Performances of two different panfungal PCRs to detect mould DNA in formalin-fixed paraffin-embedded tissue: what are the limiting factors? *BMC Infect Dis*. 2014;14(1):692.
77. Gade L, Hurst S, Balajee SA, Lockhart SR, Litvintseva AP. Detection of mucormycetes and other pathogenic fungi in formalin fixed paraffin embedded and fresh tissues using the extended region of 28S rDNA. *Med Mycol*. 2017;55(4):385-395.
78. Hammond SP, Bialek R, Milner DA, Petschnigg EM, Baden LR, Marty FM. Molecular methods to improve diagnosis and identification of mucormycosis. *J Clin Microbiol*. 2011;49(6):2151-2153.
79. Willinger B, Obradovic A, Selitsch B, Beck-Mannagetta J, Buzina W, Braun H, Apfalter P, Hirschl AM, Makristathis A, Rotter M. Detection and identification of fungi from fungus balls of the maxillary sinus by molecular techniques. *J Clin Microbiol*. 2003;41(2):581-585.
80. Eastridge BJ, Jenkins D, Flaherty S, Schiller H, Holcomb JB. Trauma system development in a theater of war: Experiences from Operation Iraqi Freedom and Operation Enduring Freedom. *J Trauma*. 2006;61(6):1366-1372; discussion 1372-1373.
81. Romanelli AM, Sutton DA, Thompson EH, Rinaldi MG, Wickes BL. Sequence-based identification of filamentous basidiomycetous fungi from clinical specimens: a cautionary note. *J Clin Microbiol*. 2010;48(3):741-752.
82. White TJ, Bruns TD, Lee SB, Taylor JW. Amplification and Direct Sequencing of Fungal Ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. *PCR Protocols: A Guide to Methods and Applications*: Academic Press; 1990. p. 315-322.
83. Bretagne S, Costa JM, Marmorat-Khuong A, Poron F, Cordonnier C, Vidaud M, Fleury-Feith J. Detection of *Aspergillus* species DNA in bronchoalveolar lavage samples by competitive PCR. *J Clin Microbiol*. 1995;33(5):1164-1168.
84. Hue FX, Huerre M, Rouffault MA, de Bievre C. Specific detection of fusarium species in blood and tissues by a PCR technique. *J Clin Microbiol*. 1999;37(8):2434-2438.
85. Bialek R, Feucht A, Aepinus C, Just-Nubling G, Robertson VJ, Knobloch J, Hohle R. Evaluation of two nested PCR assays for detection of *Histoplasma capsulatum* DNA in human tissue. *J Clin Microbiol*. 2002;40(5):1644-1647.
86. Appendix: Test performance metrics. In: Chang SM, Matchar DB, Smetana GW, Umscheid CA, editors. *Methods Guide for Medical Test Reviews*. Rockville (MD): Agency for Healthcare Research and Quality; 2012.
87. Chitasombat MN, Kontoyiannis DP. Treatment of mucormycosis in transplant patients: role of surgery and of old and new antifungal agents. *Curr Opin Infect Dis*. 2016;29(4):340-345.

88. Centers for Disease Control and Prevention. CDC/NHSN Surveillance Definitions for Specific Types of Infections. 2018.
89. Dunne JR, Hawksworth JS, Stojadinovic A, Gage F, Tadaki DK, Perdue PW, Forsberg J, Davis T, Denobile JW, Brown TS, et al. Perioperative blood transfusion in combat casualties: a pilot study. *J Trauma*. 2009;66(4 Suppl):S150-156.
90. Dunne JR, Riddle MS, Danko J, Hayden R, Petersen K. Blood transfusion is associated with infection and increased resource utilization in combat casualties. *Am Surg*. 2006;72(7):619-625; discussion 625-626.
91. Bochicchio GV, Napolitano L, Joshi M, Bochicchio K, Meyer W, Scalea TM. Outcome analysis of blood product transfusion in trauma patients: a prospective, risk-adjusted study. *World J Surg*. 2008;32(10):2185-2189.
92. Ibrahim AS, Spellberg B, Edwards J, Jr. Iron acquisition: a novel perspective on mucormycosis pathogenesis and treatment. *Curr Opin Infect Dis*. 2008;21(6):620-625.
93. Spellberg B, Edwards J, Jr., Ibrahim A. Novel perspectives on mucormycosis: pathophysiology, presentation, and management. *Clin Microbiol Rev*. 2005;18(3):556-569.
94. Ribes JA, Vanover-Sams CL, Baker DJ. Zygomycetes in human disease. *Clin Microbiol Rev*. 2000;13(2):236-301.
95. Pak J, Tucci VT, Vincent AL, Sandin RL, Greene JN. Mucormycosis in immunochallenged patients. *J Emerg Trauma Shock*. 2008;1(2):106-113.
96. Kontoyiannis DP, Chamilos G, Hassan SA, Lewis RE, Albert ND, Tarrand JJ. Increased culture recovery of Zygomycetes under physiologic temperature conditions. *Am J Clin Pathol*. 2007;127(2):208-212.
97. Ikeda K, Monden T, Kanoh T, Tsujie M, Izawa H, Haba A, Ohnishi T, Sekimoto M, Tomita N, Shiozaki H, et al. Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. *J Histochem Cytochem*. 1998;46(3):397-403.
98. Espinel-Ingroff A, Chakrabarti A, Chowdhary A, Cordoba S, Dannaoui E, Dufresne P, Fothergill A, Ghannoum M, Gonzalez GM, Guarro J, et al. Multicenter evaluation of MIC distributions for epidemiologic cutoff value definition to detect amphotericin B, posaconazole, and itraconazole resistance among the most clinically relevant species of Mucorales. *Antimicrob Agents Chemother*. 2015;59(3):1745-1750.
99. Tully CC, Romanelli AM, Sutton DA, Wickes BL, Hospenthal DR. Fatal *Actinomyces* elegans var. kuwaitiensis infection following combat trauma. *J Clin Microbiol*. 2009;47(10):3394-3399.
100. Calvano TP, Blatz PJ, Vento TJ, Wickes BL, Sutton DA, Thompson EH, White CE, Renz EM, Hospenthal DR. *Pythium aphanidermatum* infection following combat trauma. *J Clin Microbiol*. 2011;49(10):3710-3713.
101. Hospenthal DR, Chung KK, Laird K, Thompson EH, Guarro J, Renz EM, Sutton DA. *Saksenaia erythrospora* infection following combat trauma. *J Clin Microbiol*. 2011;49(10):3707-3709.
102. Radowsky JS, Strawn AA, Sherwood J, Braden A, Liston W. Invasive mucormycosis and aspergillosis in a healthy 22-year-old battle casualty: case report. *Surg Infect (Larchmt)*. 2011;12(5):397-400.
103. Paolino KM, Henry JA, Hospenthal DR, Wortmann GW, Hartzell JD. Invasive fungal infections following combat-related injury. *Mil Med*. 2012;177(6):681-685.
104. Fares Y, El-Zaatari M, Fares J, Bedrosian N, Yared N. Trauma-related infections due to cluster munitions. *J Infect Public Health*. 2013;6(6):482-486.

105. Lundy JB, Driscoll IR. Experience with proctectomy to manage combat casualties sustaining catastrophic perineal blast injury complicated by invasive mucor soft-tissue infections. *Mil Med.* 2014;179(3):e347-350.
106. Mitchell TA, Hardin MO, Murray CK, Ritchie JD, Cancio LC, Renz EM, White CE. Mucormycosis attributed mortality: A seven-year review of surgical and medical management. *Burns.* 2014;40(8):1689-1695.
107. Rodriguez CJ, Weintrob AC, Dunne JR, Weisbrod AB, Lloyd BA, Warkentien T, Malone D, Wells J, Murray CK, Bradley W, et al. Clinical relevance of mold culture positivity with and without recurrent wound necrosis following combat-related injuries. *J Trauma Acute Care Surg.* 2014;77(5):769-773.
108. Tribble DR, Rodriguez CJ, Weintrob AC, Shaikh F, Aggarwal D, Carson ML, Murray CK, Masuoka P, Infectious Disease Clinical Research Program Trauma Infectious Disease Outcomes Study G. Environmental Factors Related to Fungal Wound Contamination after Combat Trauma in Afghanistan, 2009-2011. *Emerg Infect Dis.* 2015;21(10):1759-1769.
109. Farmer AR, Murray CK, Driscoll IR, Wickes BL, Wiederhold N, Sutton DA, Sanders C, Mende K, Ennis B, Feig J, et al. Combat-related *Pythium aphanidermatum* Invasive Wound Infection: A Case Report and Discussion of the Utility of Molecular Diagnostics. *J Clin Microbiol.* 2015;53(6):1968-1975.
110. Radowsky JS, Brown TS, Lisboa FA, Rodriguez CJ, Forsberg JA, Elster EA. Serum Inflammatory Cytokine Markers of Invasive Fungal Infection in Previously Immunocompetent Battle Casualties. *Surg Infect (Larchmt).* 2015;16(5):526-532.
111. D'Alleyrand JC, Lewandowski LR, Forsberg JA, Gordon WT, Fleming ME, Mullis BH, Andersen RC, Potter BK. Combat-Related Hemipelvectomy: 14 Cases, a Review of the Literature and Lessons Learned. *J Orthop Trauma.* 2015;29(12):e493-498.
112. Akers KS, Rowan MP, Niece KL, Graybill JC, Mende K, Chung KK, Murray CK. Antifungal wound penetration of amphotericin and voriconazole in combat-related injuries: case report. *BMC Infect Dis.* 2015;15(1):184.
113. Yabes JM, White BK, Murray CK, Sanchez CJ, Mende K, Beckius ML, Zera WC, Wenke JC, Akers KS. In Vitro activity of Manuka Honey and polyhexamethylene biguanide on filamentous fungi and toxicity to human cell lines. *Med Mycol.* 2017;55(3):334-343.
114. Tribble DR, Rodriguez CJ. Combat-Related Invasive Fungal Wound Infections. *Curr Fungal Infect Rep.* 2014;8(4):277-286.
115. Rickerts V, Khot PD, Myerson D, Ko DL, Lambrecht E, Fredricks DN. Comparison of quantitative real time PCR with Sequencing and ribosomal RNA-FISH for the identification of fungi in formalin fixed, paraffin-embedded tissue specimens. *BMC Infect Dis.* 2011;11:202.

8. APPENDIX: SUPPORTING DOCUMENTATION

Figure 1. Flow Diagram of Combat Casualties with Laboratory Evidence of Fungal Infection



^a Tissue specimens for histopathology and/or wound culture were collected from any level of care (theater, LPMC, or U.S. hospitals) during initial trauma hospitalization.

^b Upon review of medical records, 64 subjects with positive histopathology were included in the molecular diagnostics analysis: 5 High Suspicion, 1 Low Suspicion, 35 Proven IFI, 22 Probable IFI, and 1 Possible IFI. The subject with the Possible IFI had specimens collected from a different wound (rather than the wound classified as the Possible IFI) that had fungal evidence, but did not meet the IFI criteria (Low Suspicion).

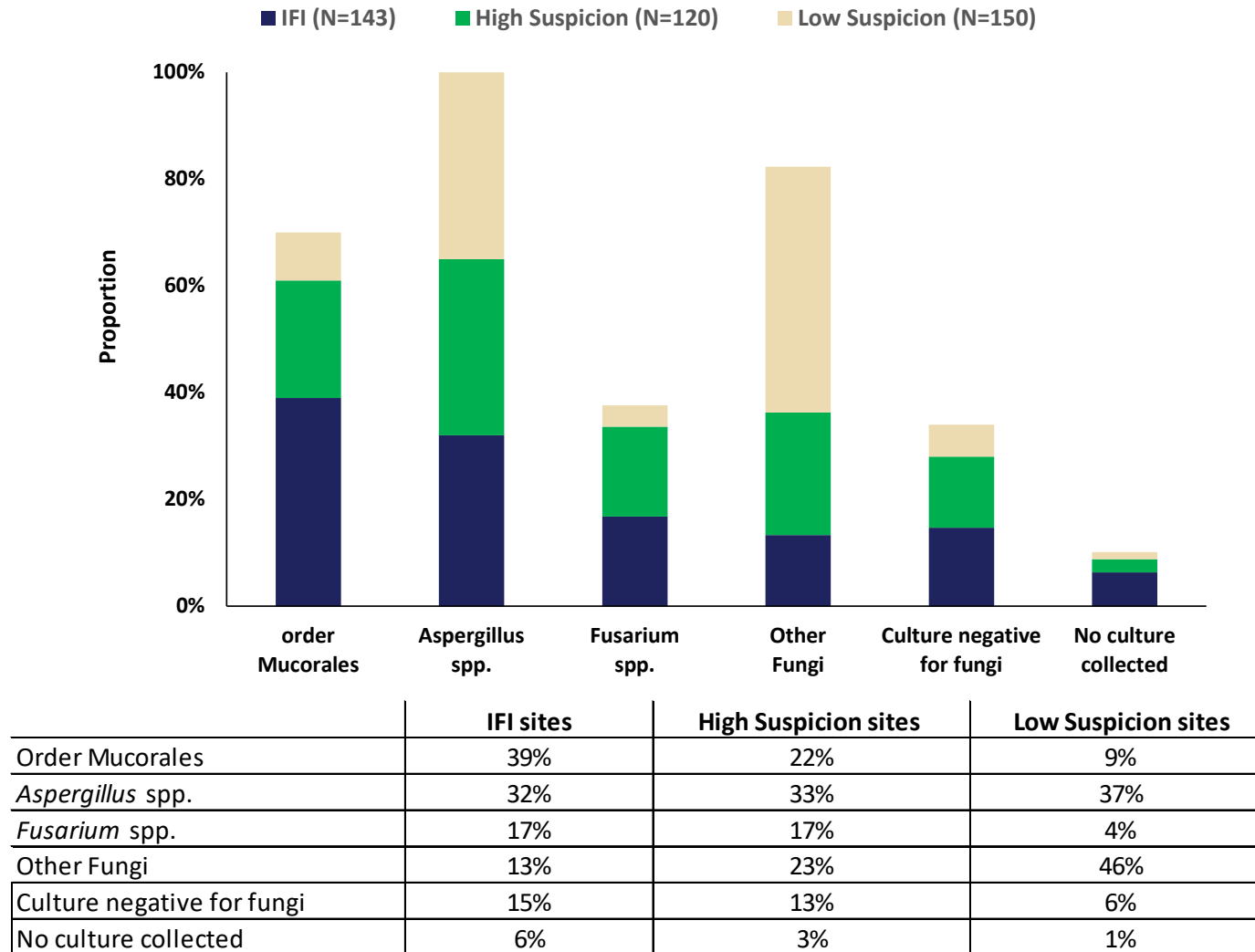
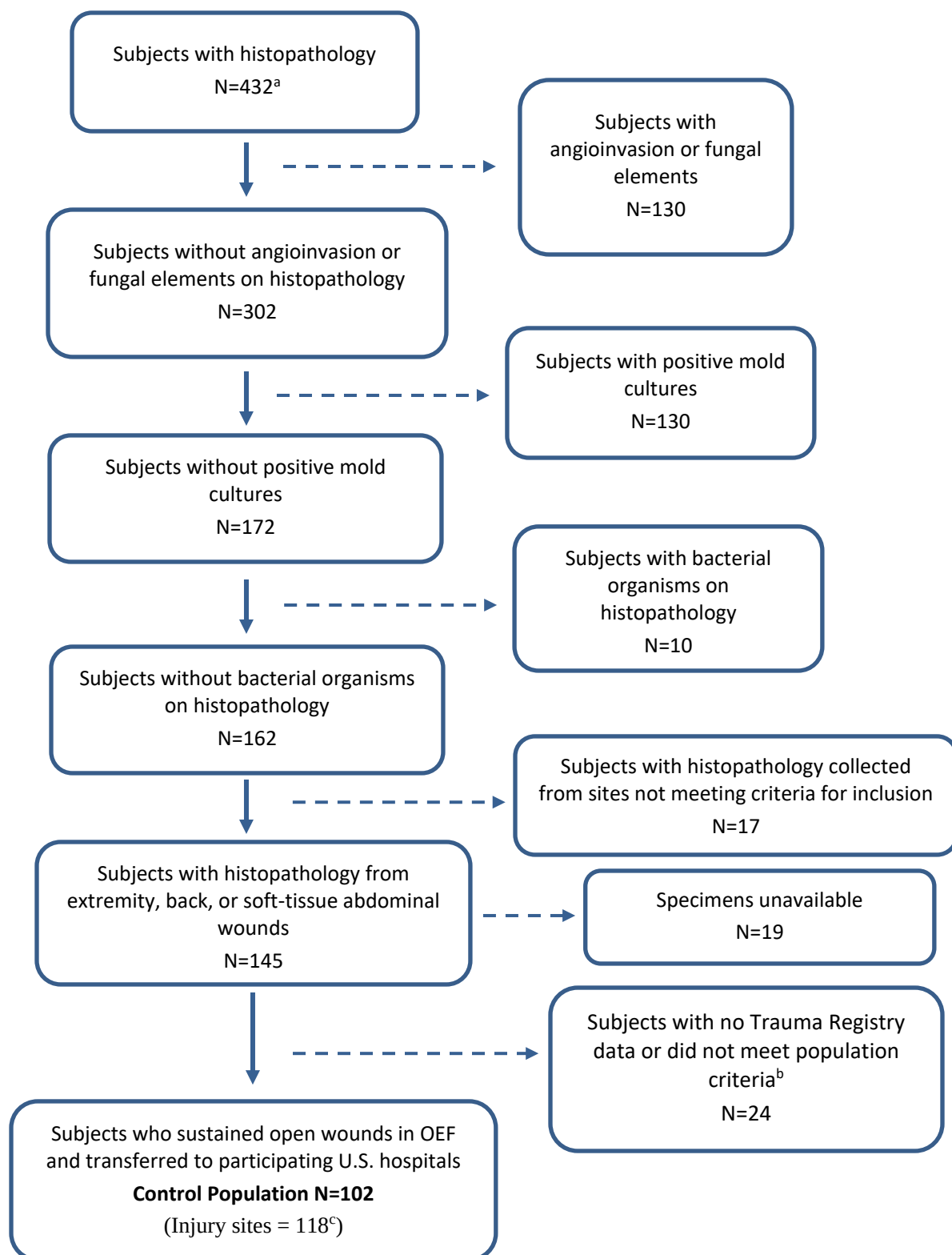
Figure 2. Culture Mycology Distribution by Fungal Classification

Figure 3. Flow Chart of Control Population for Molecular Diagnostics Evaluation of Tissue Specimens

^a A total of 374 were from the 720 subjects with open extremity wounds from Operation Enduring Freedom (OEF) and admission to the participating hospitals in the United States (**Figure 1**). There were also 58 subjects who did not meet all of these criteria (32 did not transfer to participating U.S. hospitals, 9 did not have open extremity wounds, 8 were not injured in OEF, and 9 had a combination of non-participating U.S. admission, no open extremity wounds, and/or injuries not sustained in support of OEF).

^b Population criteria included sustaining an combat-related open injury in OEF and being transferred to a participating hospital in the United States. One subject who was missing Department of Defense Trauma Registry Data was also excluded.

^c Thirty-one (26%) of the 118 control injury sites had a bacterial skin and soft-tissue infection (all classified as deep infection).

Table 1. Selected Literature Review of Trauma-related Invasive Fungal Wound Infection (IFI) Civilian Data

Reference	Number of IFI Cases	Trauma	Principal Finding
			<i>Rhizopus arrhizus</i> , <i>Absidia</i> spp., <i>Fusarium</i> spp., and unspecified fungi with nonseptate hyphae were identified
Johnson et al., 1987 ²⁰	3	Traffic accident	It was observed that gangrenous cellulitis was preceded by a change in wound appearance with progressing subcutaneous tissue and muscle necrosis
			Recommendations include extensive debridement and Amphotericin B therapy
Gordon et al., 1988 ²¹	1	Agricultural accident	Patient had extensive wounds to abdomen, perineal, and extremities
			Large plaque-like areas of gray/blackened eschar on abdominal incision and extremities noted at 48 hours
			Frozen sections identified <i>Aspergillus</i> spp., <i>Absidia</i> spp., and <i>Rhizopus</i> spp.
Patino et al., 1991 ¹⁸	8	Volcanic eruption	<i>Rhizopus arrizus</i> identified; 6 (75%) patients died
			Early diagnosis, utilizing tissue specimens and cultures, to initiate prompt treatment was advocated
Cocanour et al., 1992 ¹⁹	11	Blunt trauma (N=9); Burns (N=2)	No significant difference in injury severity between patients who died and survived;
			All deaths occurred with patients who had gangrenous cellulitis of the trunk and/or head, while those with extremity mucormycosis survived
Andresen et al., 2005 ²³	1	Tsunami	Mucormycosis observed with three non-contiguous wounds; <i>Apophysomyces elegans</i> identified from one wound

Reference	Number of IFI Cases	Trauma	Principal Finding
Roden et al., 2005 ²²	176 [literature review]	Penetrating trauma (N=60); Surgery/dressings (N=52); Burn (N=11); Traffic accident (N=5); Fall (N=5); Other (N=42)	24% of cutaneous infections deeply extended to tendon, muscle, or bone Mortality varied based on level of dissemination with cutaneous mucormycosis: localized = 10%; deep extension = 26%; disseminated = 94% Risk factors for localized cutaneous infections were female sex, no underlying condition, prior surgery, and HIV infection All patients had substantial soft-tissue loss
Moran et al., 2006 ²⁴	7	Agricultural accident (N=4); Traffic accident (N=3)	Number of debridements ranged from 4 to 20; 4 patients required amputations (1 digit-related and 3 non-digit) Mucormycetes were identified with all patients During follow-up (average 37 months), 3 patients remained on disability
Snell and Tavakoli, 2007 ²⁶	1	Tsunami	Full-thickness skin necrosis of what initially appeared to be a grade I/II pressure area on buttock; <i>Apophysomyces elegans</i> identified
Vitrat-Hincky et al., 2009 ¹²	6	Agricultural accident (N=4); Traffic accident (N=2)	Mucorales fungi isolated from 5 cases (with/without other fungi); <i>Aspergillus fumigatus</i> alone was isolated in 1 case Amputations required with 5 cases; 1 required large musculocutaneous excision Literature review found necrosis primary sign of infection (60%) and is often associated with Mucorales fungi (70%)

Reference	Number of IFI Cases	Trauma	Principal Finding
Van Sickels et al., 2011 ²⁷	1	Gunshot wounds (chest and abdomen)	Necrotic debris noted on left hepatic lobe; patient required 14 abdominal washouts and 3 liver debridements <i>Rhizopus microsporus</i> recovered from intra-abdominal wound drainage
Neblett Fanfair et al., 2012 ²⁵	13	Tornado	Case-control analysis identified penetrating trauma and occurrence of multiple wounds as risk factors; <i>Apophysomyces trapeziformis</i> identified by PCR-based assay for all cases Five (38%) deaths (fungal infection listed as primary or contributing cause of death for 3 patients)
Lanternier et al., 2012 ¹⁵	18	Traffic or agricultural accident (N=7); Leisure or gardening trauma (N=4); Burns (N=2); Other (N=5)	When compared to patient with underlying disease, mortality of patients with trauma-associated IFI was lower (11% versus 32-60%) 95% of patients with cutaneous infections had positive cultures
Gomez-Camarasa et al., 2013 ²⁸	1	Agricultural accident	Patient had history of uncontrolled diabetes mellitus Necrotic eschar and cellulitis observed 10 days post-injury Molecular sequencing identified <i>Saksenaea vasiformis</i>

Reference	Number of IFI Cases	Trauma	Principal Finding
Lelievre et al., 2014 ¹³	16	Traffic accident (N=6); Agricultural accident (N=3); Explosion (N=2); Minor injury or other (N=5)	Symptoms occurred median of 8 days post-injury; necrosis was observed in 10 (63%) patients; 2 (13%) deaths reported All cases had fungi from order Mucorales grown in culture; <i>Rhizopus</i> spp. were predominant (13 patients; 81%); <i>Mucor</i> , <i>Saksenaea</i> , and <i>Apophysomyces</i> spp also recovered from trauma patients, but not from patients with other forms of mucormycosis
Ingram et al., 2014 ²⁹	9	Traffic accident (N=7); Other (N=2)	Median time to diagnosis from onset of symptoms was 11.5 days; 4 (44%) patients had deep infections; 1 (11%) death was reported Fungi were identified at species level for all patients using culture and molecular identification; <i>Rhizopus microsporus</i> . recovered from 4 (44%) patients
Coronel-Perez et al., 2015 ³⁰	1	Insect bite/sting	<i>Saksenaea vasiformis</i> identified as the etiologic agent
Kennedy et al., 2016 ¹⁶	17	Traffic accident, burn, and Tsunami (numbers unspecified)	All had mucormycetes identified and 53% had disseminated infections Patients with trauma were significantly more likely to have a non- <i>Rhizopus</i> spp. infection compared to patients with other predisposing factors or co-morbidities
Chander et al., 2017 ¹⁷	5	Intramuscular injection (N=4); Other (N=1)	Trauma was not significantly associated with mortality Except for 1 patient with diabetes mellitus diagnosed during treatment, the patients were immune competent with no obvious risk factors <i>Saksenaea erythrospora</i> was identified from all infections using molecular methods

Table 2. Landstuhl Regional Medical Center (LRMC) Blast Protocol Criteria for the Screening of Patients for Invasive Fungal Infections (IFI)³¹

-
1. The trauma team should screen for IFI in all patients who sustained bilateral lower extremity amputation secondary to improvised explosive device or blast injury
- OR*
2. The trauma team should also strongly consider IFI screening in any patient with any one of the following conditions:
 - a. Large amputation burden: >1 amputation, single amputation above mid-femur, or single amputation in conjunction with significant perineal soft tissue injury.
 - b. More than expected soft tissue necrosis (either by rate of progression or progression outside zone of injury) based on communication with downrange providers confirming the interval since last debridement and that prior debridement was to viable tissue.
 - c. Extensive wound contamination (as evidenced by continued gross contamination of wound at initial LRMC washout)
 - d. Significant revision of amputation level required at LRMC
-

Table 3. Literature Review of Combat-related Invasive Fungal Wound Infection (IFI) Clinical Research^a

Reference	Number of IFI Cases	Principal Finding
Tully et al., 2009 ⁹⁹	1	First case of invasive mucormycosis secondary to <i>Actinomucor elegans</i> infection. Mucormycosis should be considered in cases with recurrent, necrotic wounds when other risk factors (e.g., open traumatic wound with resulting immune suppression) are also present as it is likely fatal until treated early and appropriately.
Calvano et al., 2011 ¹⁰⁰	1	Rare infection with a plant fungal pathogen (<i>Pythium aphanidermatum</i>).
Evriviades et al., 2011 ³	Unspecified	U.K. military experience suggesting risk factors considered for management decisions included: 1) injured in southern Afghanistan; 2) dismounted blast injury; 3) extensive wounds/amputations contaminated with environmental debris; and 4) required massive blood product transfusions (>8 units) within 24 hours of injury.
Hospenthal et al., 2011 ¹⁰¹	1	First report of newly described species, <i>Saksenaea erythrospora</i> , as a cause of a fatal human infection.
Radowsky et al., 2011 ¹⁰²	1	Early diagnosis and testing for invasive fungal infections is critical for effective management.
Paolino et al., 2012 ¹⁰³	6	Walter Reed Army Medical Center case experience reporting invasive fungal infections are a rare complication of combat wounds from Iraq, but associated with significant morbidity
Warkentien et al., 2012 ²	37	Outbreak investigation of emerging combat trauma-related invasive fungal infections, which lead to significant morbidity. Early identification, aggressive surgical debridement, and antifungal therapy are critical for effective management.
Barsoumian et al., 2013 ³²	None	Dakin's solution exhibits better dose dependent toxicity and efficacy compared to mafenide acetate and Amphotericin B with regards to topical application.
Fares et al., 2013 ¹⁰⁴	9	Blast wounds were contaminated with fungi and heat and moisture resulting from heavy, dirty clothing creating ideal habitat for fungal growth and proliferation
Blyth et al., 2013 ³⁴	15 [burn patients]	Fungal wound colonization or infection was associated with higher total body surface area burned. Fungal screening assays [(1,3)- β -D-glucan and galactomannan] were not significantly associated with fungal wound infection, colonization, or isolation of specific fungi; thus, these assays should be used with caution in burn patients.
Lewandowski et al., 2013 ³³	3	The use of Dakin's solution, along with surgical management and antifungal therapy, was successful at treating angioinvasive fungal infections.

Reference	Number of IFI Cases	Principal Finding
Lloyd et al., 2014 ³¹	74 ^b	Implementation of a local clinical practice guideline resulted in earlier diagnosis and initiation of treatment for IFIs. Nonetheless, no difference in clinical outcome was observed.
Rodriguez et al., 2014 ⁸	76 ^b	Case-control analysis determined that blast injuries sustained while dismounted, resulting in above knee amputations and requiring large-volume blood product transfusions (>20 units) within 24 hours of injury are independent risk factors for the development of IFIs
Lundy and Driscoll, 2014 ¹⁰⁵	2	Provides first description of management of complex pelvic/perineal soft-tissue injuries complicated with IFI through use of proctectomy.
Mitchell et al., 2014 ¹⁰⁶	11 [burn patients]	Aggressive surgical treatment is necessary to manage invasive mucormycosis. Implementation of a standard protocol or surveillance cultures should be considered.
Rodriguez et al., 2014 ¹⁰⁷	77 ^b cases plus 19 with fungal growth and no necrosis	In absence of recurrently necrotic wounds, fungal growth on wound cultures among patients with blast trauma does not require systemic antifungal therapy.
Weintrob et al., 2015 ⁴	77 ^b	Trauma-related IFI classification scheme an effective tool for clinical and epidemiological surveillance/research.
Tribble et al., 2015 ¹⁰⁸	71 ^b	Being injured in southern Afghanistan (versus eastern Afghanistan) was significantly associated with fungal contamination of wounds. Region characterized by lower elevation, warmer temperature, and greater isothermality.
Warkentien et al., 2015 ¹⁰	82 wounds ^b	Characteristics of wounds with an IFI were compared to those with only a bacterial infection or no infection. The IFI wounds frequently grew bacteria, including multidrug-resistant organisms. IFI wounds had significantly longer time to wound closure versus comparator groups. Wounds with growth of fungi from the order <i>Mucorales</i> had significantly longer time to wound closure compared to IFI wounds without <i>Mucorales</i> growth.
Farmer et al., 2015 ¹⁰⁹	1 [burn / blast patient]	Use of PCR-based methods to enable the identification of a rare pathogen (<i>Pythium aphanidermatum</i>)

Reference	Number of IFI Cases	Principal Finding
Radowsky et al., 2015 ¹¹⁰	9	Elevation in serum inflammatory cytokines from 1 st and 2 nd debridements were associated with IFIs when compared to nine matched controls. Further studies are needed to evaluate the potential of rapid serum testing to aid in IFI diagnosis and initiation of treatment.
D'Alleyrand et al., 2015 ¹¹¹	12 extremities	Examination of characteristics and management of 14 combat-related hemipelvectomies; 86% of the extremities had a fungal infection
Akers et al., 2015 ¹¹²	2	Systemic pharmacokinetics and wound effluent antifungal concentrations were examined. High variability within and between patients was identified. Free Amphotericin B was not present in wound effluent despite sufficient concentrations in circulating plasma. Voriconazole was detected in wound effluent but at varying concentrations (both above and below minimum inhibitory concentrations for Mucorales).
Heaton et al., 2016 ³⁵	66 ^b	Histopathological techniques for IFI diagnosis were examined. Periodic acid-Schiff and Gomori methenamine silver (GMS) were 84% concordant, but GMS had lower false negative rate, suggesting it may be more sensitive. While frozen sections had high specificity, sensitivity was 60%, so it should not be used as a standalone method for IFI diagnosis.
Lewandowski et al., 2016 ⁹	112 wounds ^b	Case-control analysis determined that IFI wounds result in significantly more proximal changes in amputation level, surgical procedures, and a longer time to wound closure, further worsened by co-infection with multidrug-resistant Gram-negative bacteria. These findings confirm the negative impact of IFIs on patient recovery and wound healing.
Yabes et al., 2017 ¹¹³	None	Antifungal activity and cytotoxicity of Manuka honey and polyhexamethylene biguanide was assessed with 13 clinical fungal isolates. Antifungal activity of both agents was correlated more closely with exposure time versus concentration. Both agents also exert <i>in vitro</i> toxicity on human cells which may limit therapeutic use.

^a modified from Tribble and Rodriguez (2014)¹¹⁴

^b analyses involves the same overall TIDOS invasive fungal wound infection (IFI) population of 77 patients; includes the initial 37 IFI patients detailed in Warkentien et al., 2012²

Table 4. Selected Literature Review of PCR Findings with Formalin-Fixed Paraffin-Embedded Tissue

Reference	Assay	Population / Study Type	Specimen Number	Sensitivity (%)	Specificity (%)	Comments
Willinger et al., 2003 ⁷⁹	PCR targeting 28s region of ribosomal DNA	Immune competency not specified	81	87.7	Not specified	Two detection methods were utilized with sensitivity higher for sequencing (50.7%) versus dot blot hybridization (36.6%) Among specimens with results using both sequencing and dot blot hybridization, 20% (3 of 15 specimens) of findings were discordant
Bialek et al., 2005 ⁶⁷	Two semi-nested PCRs targeting (1) mucormycetes and (2) <i>Aspergillus fumigatus</i>	Immune competency not specified	52	61	Not specified	Concomitant infections were identified using the 2 PCR assays PCR failed to detect fungal DNA in 16 specimens with an infection identified by histopathology (36% false negative)
Hata et al., 2008 ⁷²	Real-time PCR targeting mucormycete cytochrome b gene	Immune competency not specified	81	56	100	Specific to 6 species of mucormycetes No cross-reactivity was demonstrated with tested species of other fungi, yeasts, or bacteria

Reference	Assay	Population / Study Type	Specimen Number	Sensitivity (%)	Specificity (%)	Comments
Lau et al., 2007 ⁷⁰	Panfungal PCR targeting ITS1	Immune competency not specified	16 [culture proven] 22 [histology proven]	Culture proven = 93.6 Histology proven = 64.3	Not specified	The PCR performed well on specimens where fungal elements were visualized but not grown There is potential benefit to using PCR in combination with conventional laboratory testing
Munoz-Cadavid et al., 2010 ⁷¹	Panfungal PCR targeting ITS3 and ITS4	Immune competency not specified	51	53 to 86	Not specified	Sensitivity was tested using 5 different DNA extraction protocols 6 animal specimens and 24 human mock tissues were also assessed and sensitivity for all 81 specimens was 60.5 to 91%
Rickerts et al., 2011 ¹¹⁵	Quantitative PCR targeting ribosomal RNA (ITS-2 and 28s region)	Immune compromised or not specified	40	70	Not specified	The PCR was most successful in samples with invasive yeast infection and in samples with septate fungal hyphae (80% sensitivity)
Hammond et al., 2011 ⁷⁸	Semi-nested PCR targeting 18s region of ribosomal DNA of mucormycetes	Immune compromised	27	81	Not specified	Among 12 specimens with positive cultures, PCR was concordant at genus level with 9 PCR allowed for genus level identification in 12 specimens with negative cultures

Reference	Assay	Population / Study Type	Specimen Number	Sensitivity (%)	Specificity (%)	Comments
Buitrago et al., 2013 ⁷³	Panfungal PCR targeting ITS1 and ITS2	Immune compromised, trauma, and other not specified	89	89	Not specified	PCR sensitivity was higher than sensitivity calculated for culture (56%)
						PCR detected DNA in 80% of specimens with negative cultures
						PCR failed to detect fungal DNA in 3 specimens with histopathological evidence
						Mucorales fungi associated with skin / subcutaneous samples
Bernal-Martinez et al., 2013 ⁶⁶	Multiplex real-time PCR targeting ITS1 and ITS2	Immune compromised, trauma, or not specified	12	100 [for strains containing species that were target]	Not specified	DNA from negative controls was not amplified The assay detected all infections caused by <i>Rhizopus</i> spp. or <i>Mucor</i> spp.; only infections caused by other fungal species were not detected
Babouee Flury et al., 2014 ⁷⁶	45-cycle panfungal PCR targeting ITS2 and ITS1-2 regions	Immune competency not specified	27	ITS2 = 53.8; ITS1-2 = 38	Not specified	Amplification and identification with 14 specimens; organisms identified corresponded to culture findings and PCR from fresh tissue

Reference	Assay	Population / Study Type	Specimen Number	Sensitivity (%)	Specificity (%)	Comments
Salehi et al., 2016 ⁶⁹	Real-time quantitative PCR targeting ITS2	Immune competency not specified	102	64	Not specified	DNA extraction method had 94% sensitivity; no fungal DNA amplified from 6 control specimens. 80% concordance between PCR and histopathology qPCR failed to detect fungal DNA in 9 specimens likely due to DNA destruction prior to paraffin was embedding
Drogari- Apiranthitou et al., 2016 ⁵²	Semi-nested PCR targeting mitochondrial <i>Aspergillus</i> spp. DNA and 18s region of ribosomal DNA of mucormycetes	Immune compromised or surgical patient	30	79.3	100	PCR failed to detect fungal DNA in 7 specimens with histopathological / culture evidence Mucormycetes were more frequent in histology specimens with fungal hyphae than <i>Aspergillus</i> spp.
Springer et al., 2016 ⁵¹	Real-time probe-based PCR targeting 18s region of ribosomal Mucorales DNA	Immune compromised or trauma	12	91	100	PCR failed to detect fungal DNA in one specimen

Reference	Assay	Population / Study Type	Specimen Number	Sensitivity (%)	Specificity (%)	Comments
Gade et al., 2017 ⁷⁷	PCR targeting 28s region of ribosomal DNA	Unspecified; specimens were submitted to CDC laboratory as part of routine surveillance or outbreak investigations	33	Not specified	Not specified	9 specimens were from IFI patients injured by the Joplin tornado When compared to PCR targeting the ITS1 and ITS2, PCR amplification and sequencing of the 28s ribosomal region was more sensitive

ITS – internal transcribed spacer

Table 5. Characteristics of Patients with Combat-Related Invasive Fungal Wound Infections and Laboratory Fungal Evidence

Characteristics, No. (%)	IFI (N=94)	High Suspicion (N=61) ^a	P-value ^b	Low Suspicion (N=91)	P-value ^c
Blast Injury	92 (97.9)	61 (100)	0.520	89 (97.8)	1.000
Dismounted injury ^d	73 (94.8)	51 (94.4)	1.000	80 (95.2)	1.000
<i>Injury Severity Score, median (IQR)</i>	40 (33, 50)	38 (30, 45)	0.262	33 (27, 42)	<0.001
1-9 (mild)	0	0	0.063	0	0.059
10-15 (moderate)	2 (2.1)	0		2 (2.2)	
16-25 (severe)	5 (5.3)	9 (14.8)		14 (15.4)	
≥26 (critical)	87 (92.6)	52 (85.3)		75 (82.4)	
<i>Blood units 24 hours post-injury, median (IQR)^e</i>	31 (21, 43)	21 (15, 32)	0.003	17 (12, 24)	<0.001
1-9	4 (4.3)	5 (8.2)	0.005	15 (17.2)	<0.001
10-20	18 (19.4)	25 (41.0)		42 (48.3)	
>20	71 (76.3)	31 (50.8)		30 (34.5)	
Traumatic amputations	64 (68.1)	48 (78.7)	0.150	73 (80.2)	0.060
LRMC SOFA, median (IQR)	11 (7, 13)	8 (4, 13)	0.028	6 (2, 9)	<0.001
U.S. hospital SOFA, median (IQR)	7 (2, 11)	4 (1, 8)	0.022	1 (0, 6)	<0.001
<i>Mechanical ventilation</i>			0.132		<0.001
None	14 (14.9)	15 (24.6)		40 (44.0)	
LRMC only	18 (19.2)	14 (23.0)		25 (27.5)	
LRMC and U.S. hospital ≤1 week	62 (66.0)	31 (50.8)		26 (28.6)	
LRMC and U.S. hospital >1 week	0	1 (1.6)		0	

IQR – interquartile range; LRMC – Landstuhl Regional Medical Center; SOFA – sequential organ failure assessment

^a One subject classified as a High Suspicion died within 24 hours of laboratory fungal evidence being collected, precluding classification as an IFI

^b P-value compares characteristics between IFI and High Suspicion groups

^c P-value compares characteristics between IFI and Low Suspicion group

^d Mounted status is missing for 17 IFI subjects, 7 High Suspicion subjects, and 7 Low Suspicion subjects. Percentages and p-values based on total minus missing.

^e Blood information is missing for 1 IFI subject, and 4 Low Suspicion subjects. Percentages and p-values based on total minus missing.

Table 6. Characteristics of Patients Stratified by Laboratory Evidence of Fungal Infection and Dismounted Complex Blast Injury (DCBI) Status^a

Characteristics, No. (%)	DCBI		P-value ^d	IFI or HS without DCBI (N=74) ^e	P-value ^f
	With IFI or HS (N=81) ^b	Without IFI or HS (N=81) ^c			
Blast Injury	81 (100)	81 (100)	NA	72 (97.3)	0.226
Dismounted injury	81 (100)	81 (100)	NA	43 (86.0) ^g	0.001
<i>Injury Severity Score, median (IQR)</i>	38 (33, 50)	33 (30, 38)	0.001	35 (30, 45)	0.048
1-9 (mild)	0	0	NA	0	<0.001
10-15 (moderate)	0	0		2 (2.7)	
16-25 (severe)	0	0		14 (18.9)	
≥26 (critical)	81 (100)	81 (100)		58 (78.4)	
<i>Blood units 24 hrs post-injury, median (IQR)^h</i>	30 (19, 44)	20 (15, 28)	<0.001	24 (16, 36)	0.042
1-9	4 (4.9)	6 (7.4)	0.005	5 (6.8)	0.199
10-20	18 (22.2)	36 (44.4)		25 (34.2)	
>20	59 (72.8)	39 (48.2)		43 (58.9)	
<i>Traumatic amputationsⁱ</i>					
Above knee	81 (100)	81 (100)	NA	32 (43.2)	<0.001
Bilateral amputation	55 (67.9)	56 (69.1)	0.866	17 (23.0)	<0.001
Amputation plus injury to opposite limb ^j	81 (100)	81 (100)	NA	27 (36.5)	<0.001
Perineal, GU injury, or abdominal injury	81 (100)	81 (100)	NA	62 (83.8)	<0.001
Colostomy	27 (33.3)	12 (14.8)	0.006	12 (16.2)	0.014
LRMC SOFA, median (IQR)	9 (6, 13)	6 (3, 9)	<0.001	10 (5, 13)	0.853
U.S. hospital SOFA, median (IQR)	7 (2, 10)	2 (0, 6)	<0.001	6 (1, 10)	0.581
<i>Mechanical ventilation</i>			0.001		0.760
None	16 (19.8)	30 (37.0)		13 (17.6)	
LRMC only	15 (18.5)	24 (29.6)		17 (23.0)	
LRMC and U.S. hospital ≤1 week	49 (60.5)	27 (33.3)		44 (59.5)	
LRMC and U.S. hospital >1 week	1 (1.2)	0		0	

GU – genitourinary injury; IQR – interquartile range; LRMC – Landstuhl Regional Medical Center; SOFA – sequential organ failure assessment

^a Population is a subset of the 720 subjects with tissue specimens/wound cultures collected for analysis in Figure 1. A total of 162 subjects met the criteria of a DCBI.

^b Includes 49 IFI and 32 High Suspicion (HS) subjects

^c Includes 38 subjects classified as Low Suspicion and 43 subjects without laboratory evidence of a fungal infection

^d P-values compares the DCBI patients with and without IFI or HS

^e Includes 45 IFI and 29 HS subjects

^f P-value compares the IFI/HS patients (those with DCBI and those without)

^g Mounted Status missing for 24 subjects. Percentages and p-value based on total minus missing.

^h Blood information missing for one subject. Percentages and p-value based on total minus missing.

ⁱ Subjects may have more than one type of traumatic amputation so categories are not mutually exclusive

^j Severe injury to opposite limb is defined using Abbreviated Injury Scale (AIS) postdot injury score ≥ 3 . Severe injury to the opposite limb also includes amputations, excluding digits.

Table 7. Characteristics of Subjects with Tissue Specimens Analyzed by PCR-based Assays

Characteristics, No. (%)	Subjects with Positive Histopathology (N=64)	Controls (N=102)	P-value
Operation Enduring Freedom	64 (100)	102 (100)	NA
Blast Injury	62 (96.9)	82 (80.4)	0.002
Dismounted injury ^a	49 (94.2)	76 (85.4)	0.168
<i>Injury Severity Score, median (IQR)</i>	42 (33, 57)	25.5 (18, 33)	<0.001
1-9 (mild)	0	3 (2.9)	<0.001
10-15 (moderate)	0	11 (10.8)	
16-25 (severe)	2 (3.1)	37 (36.3)	
≥26 (critical)	62 (96.9)	51 (50.0)	
<i>Blood units 24 hours post-injury, median (IQR)</i>	32 (23, 44)	10 (5, 19)	<0.001
Zero	0	2 (2.2) ^b	<0.001
1-9	4 (6.3)	40 (43.0)	
10-20	6 (9.4)	32 (34.4)	
>20	54 (84.4)	19 (20.4)	
<i>Traumatic amputations^c</i>			
Above knee	38 (59.4)	21 (20.6)	<0.001
Bilateral amputation	9 (14.1)	5 (4.9)	0.039
Amputation plus serious injury to opposite limb	31 (48.4)	16 (15.7)	<0.001
Perineal, GU injury, or abdominal injury	61 (95.3)	63 (61.8)	<0.001
Colostomy	20 (31.3)	3 (2.9)	<0.001
LRMC SOFA, median (IQR)	11 (6, 13)	3 (2, 8)	<0.001
U.S. hospital SOFA, median (IQR)	8 (4, 12)	1 (0, 2)	<0.001
<i>Mechanical ventilation</i>			<0.001
None	11 (17.2)	63 (61.8)	
LRMC only	7 (10.9)	20 (19.6)	
LRMC and U.S. hospital ≤1 week	46 (71.9)	17 (16.7)	
LRMC and U.S. hospital >1 week	0	2 (2.0)	

GU – genitourinary injury; IQR – interquartile range; LRMC – Landstuhl Regional Medical Center; SOFA – sequential organ failure assessment

^a Mounted status is missing for 12 subjects with positive histopathology and 13 control subjects. Percentages and p-value based on total minus missing.

^b Blood information is missing for 9 control subjects. Percentages and p-value based on total minus missing.

^c Subjects may have more than one type of traumatic amputation so categories are not mutually exclusive

Table 8. Frequency of Identification of Growth of Filamentous Fungi with Culture^a

Culture Findings, No. (%)^b	Sites with Angioinvasion (N=48)	Sites with Nonvascular tissue invasion (N=47)
Cultures sent	44	43
No fungal growth	7 (15.9)	12 (27.9)
Order Mucorales	20 (45.5)	11 (25.6)
<i>Saksenaia</i> spp.	6 (13.6)	2 (4.7)
<i>Rhizopus</i> spp.	2 (4.5)	2 (4.7)
<i>Apophysomyces</i> spp.	1 (2.3)	0
<i>Actinomucor</i> spp.	3 (6.8)	0
<i>Aspergillus</i> spp.	13 (29.5)	11 (25.6)
<i>A. terreus</i>	5 (11.4)	5 (11.6)
<i>A. flavus</i>	6 (13.6)	6 (14.0)
<i>A. fumigatus</i>	1 (2.3)	1 (2.3)
<i>Other species</i>	3 (6.8)	4 (9.3)
<i>Fusarium</i> spp.	11 (25.0)	4 (9.3)
Combinations		
Mucorales plus <i>Aspergillus</i> spp.	3 (6.8)	1 (2.3)
<i>Aspergillus</i> plus <i>Fusarium</i> spp.	1 (2.3)	0
Mucorales plus <i>Fusarium</i> spp.	4 (9.1)	1 (2.3)
Mucorales plus <i>Aspergillus</i> pp. plus <i>Fusarium</i> spp.	2 (4.5)	0

^a By definition, control specimens did not have any histopathological evidence of a fungal infection

^b Categories are not mutually exclusive

Table 9. Frequency of Identification of Filamentous Fungal DNA with PCR-based Assay Based Upon Histopathological Evidence^a

PCR Findings, No. (%) ^b	Sites with Angioinvasion (N=48)	Sites with Nonvascular tissue invasion (N=47)
No fungal DNA detected	8 (16.7)	27 (57.5)
Order Mucorales	36 (75.0)	10 (21.3)
<i>Saksenaea</i> spp.	26 (54.2)	4 (8.5)
<i>Rhizopus</i> spp.	2 (4.2)	1 (2.1)
<i>Apophysomyces</i> spp.	5 (10.4)	3 (6.4)
<i>Actinomucor</i> spp.	5 (10.4)	1 (2.1)
<i>Aspergillus</i> spp.	7 (14.6)	4 (8.5)
<i>A. terreus</i>	1 (2.1)	1 (2.1)
<i>A. flavus</i>	4 (8.3)	3 (6.4)
<i>A. tamarii/vitricola/penicilliioides/gracilis</i>	3 (6.3)	0
<i>Fusarium</i> spp.	2 (4.2)	4 (8.5)
Combinations		
Mucorales plus <i>Aspergillus</i> spp.	4 (8.3)	0
<i>Aspergillus</i> plus <i>Fusarium</i> spp.	1 (2.1)	1 (2.1)
Mucorales plus <i>Fusarium</i> spp.	1 (2.1)	0

^a By definition, control specimens did not have any histopathological evidence of a fungal infection

^b Categories are not mutually exclusive

Table 10. Filamentous Fungi Positivity Rate for Injury Sites with Both Positive Histopathology (Angioinvasion or Nonvascular Tissue Invasion) and Cultures Collected^{a,b}

Growth of Filamentous Fungi, No. (%)	Culture Result	PCR Results	Kappa Coefficient
Cultures collected at LRMC or U.S. hospitals, Sites No.	87	87	
<i>Growth of fungi from cultures at LRMC or U.S hospitals:</i>			
No fungi	19 (21.8)	30 (34.5)	0.248 (0.039, 0.457)
Any fungi	68 (78.2)	57 (65.5)	
Fungi identified at genus level ^c	63 (72.4)	57 (65.5)	
Order Mucorales	31 (35.6)	43 (49.4)	0.262 (0.067, 0.457)
<i>Saksenaea</i> spp.	8 (9.2)	31 (35.6)	0.129 (-0.039, 0.298)
<i>Aspergillus</i> spp.	24 (27.6)	12 (13.8)	0.387 (0.169, 0.605)
<i>Fusarium</i> spp.	15 (17.2)	6 (6.9)	0.208 (-0.050, 0.466)
Cultures collected only at U.S. hospitals only, Sites No.	86	86	
<i>Growth of fungi from cultures at U.S hospitals only:</i>			
No fungi	24 (27.9)	29 (33.7)	0.267 (0.053, 0.481)
Any fungi	62 (72.1)	57 (66.3)	
Fungi identified at genus level ^c	57 (66.3)	57 (66.3)	
Order Mucorales	29 (33.7)	43 (50.0)	0.302 (0.112, 0.493)
<i>Saksenaea</i> spp.	8 (9.3)	31 (36.1)	0.127 (-0.041, 0.296)
<i>Aspergillus</i> spp.	21 (24.4)	12 (14.0)	0.447 (0.220, 0.675)
<i>Fusarium</i> spp.	13 (15.1)	6 (7.0)	0.127 (-0.127, 0.381)
Cultures collected ±24 hours of histopathology specimen collection, Sites No.	77	77	
No fungi	22 (28.6)	29 (37.7)	0.332 (0.115, 0.549)
Any fungi	55 (71.4)	48 (62.3)	
Fungi identified at genus level ^c	50 (64.9)	48 (62.3)	
Order Mucorales	25 (32.5)	37 (48.1)	0.421 (0.227, 0.614)
<i>Saksenaea</i> spp.	7 (9.1)	26 (33.8)	0.187 (-0.004, 0.377)
<i>Aspergillus</i> spp.	14 (18.2)	8 (10.4)	0.371 (0.093, 0.650)
<i>Fusarium</i> spp.	8 (10.4)	3 (3.9)	-0.060 (-0.112, -0.008)

LRMC – Landstuhl Regional Medical Center

^a By definition, control specimens did not have any histopathology and culture evidence of a fungal infection

^b The PCR specimens were collected only from U.S. hospitals; however, cultures were collected at various time points and at different facilities. Analysis is restricted to subsets of injury sites

with tissue specimens for histopathology and cultures collected from the same injury site at LRMC or U.S. hospitals (N=87), at U.S. hospitals (N=86), and within 24 hours of histopathology specimen collection (N=77). Categories are independent of each other.

^c Excludes *Mycelia sterilia* and fungi classified as ‘not otherwise specified’ or ‘zygomycetes’

Table 11. Filamentous Fungi Positivity Rate for Sites with Both Angioinvasion and Cultures Collected^{a,b}

Growth of Filamentous Fungi, No. (%)	Culture Result	PCR Results	Kappa Coefficient
Cultures collected at LRMC or U.S. hospitals, Sites No.	44	44	
<i>Growth of fungi from cultures at LRMC or U.S hospitals:</i>			
No fungi	7 (15.9)	7 (15.9)	-0.019 (-0.304, 0.266)
Any fungi	37 (84.1)	37 (84.1)	
Fungi identified at genus level ^c	35 (79.6)	37 (84.1)	
Order Mucorales	20 (45.5)	33 (75.0)	0.087 (-0.154, 0.327)
<i>Saksenaea</i> spp.	6 (13.6)	25 (56.8)	0.049 (-0.130, 0.228)
<i>Aspergillus</i> spp.	13 (29.6)	7 (15.9)	0.370 (0.069, 0.670)
<i>Fusarium</i> spp.	11 (25.0)	2 (4.6)	0.250 (-0.039, 0.539)
Cultures collected only at U.S. hospitals only, Sites No.	44	44	
<i>Growth of fungi from cultures at U.S hospitals only:</i>			
No fungi	7 (15.9)	7 (15.9)	-0.019 (-0.304, 0.266)
Any fungi	37 (84.1)	37 (84.1)	
Fungi identified at genus level ^c	35 (79.6)	37 (84.1)	
Order Mucorales	20 (45.5)	33 (75.0)	0.087 (-0.154, 0.327)
<i>Saksenaea</i> spp.	6 (13.6)	25 (56.8)	0.049 (-0.130, 0.228)
<i>Aspergillus</i> spp.	12 (27.3)	7 (15.9)	0.407 (0.099, 0.716)
<i>Fusarium</i> spp.	11 (25.0)	2 (4.6)	0.250 (-0.039, 0.539)
Cultures collected ±24 hours of histopathology specimen collection, Sites No.	41	41	
No fungi	6 (14.6)	8 (19.5) ^d	-0.029 (-0.316, 0.257)
Any fungi	35 (85.4)	33 (80.5)	
Fungi identified at genus level ^c	32 (78.1)	33 (80.5)	
Order Mucorales	18 (43.9)	28 (68.3)	0.253 (-0.004, 0.510)
<i>Saksenaea</i> spp.	5 (12.2)	20 (48.8)	0.155 (-0.049, 0.359)
<i>Aspergillus</i> spp.	6 (14.6)	5 (12.2)	0.476 (0.084, 0.868)
<i>Fusarium</i> spp.	6 (14.6)	1 (2.4)	-0.044 (-0.119, 0.032)

LRMC – Landstuhl Regional Medical Center

^a By definition, control specimens did not have angioinvasion and culture evidence of a fungal infection

^b The PCR specimens were collected only from U.S. hospitals; however, cultures were collected at various time points and at different facilities. Analysis is restricted to injury sites with

observed angioinvasion and cultures collected from the same injury site LRMC or U.S. hospitals (N=44), only at U.S. hospitals (N=44), and within 24 hours of histopathology specimen collection (N=41). Categories are independent of each other.

^c Excludes *Mycelia sterilia* and fungi classified as ‘not otherwise specified’ or ‘zygomycetes’

^d Injury sites may have more than one specimen collected and the results are collapsed to a site level. One subject with two specimens from one injury site had one of the specimens excluded after the restriction of having a culture collected within 24 hours applied. The specimen excluded was positive for fungal growth (*Aspergillus* spp. and *Saksenea* spp), while the included specimen was negative.

Table 12. Comparison of PCR-based Assay Versus Histopathology for Identification of Fungus in Formalin-fixed Paraffin-embedded Tissue^{a,b}

<u>PCR assay</u>	<u>Histopathology</u>		Total
	Negative for fungal elements (Controls)	Positive for fungal elements	
Negative for fungal DNA	117	35	152
Positive for fungal DNA^c	1 ^d	60	61
Total	118	95	213

^a Includes both angioinvasion and fungal elements observed on histopathology without invasion

^b Kappa coefficient for comparison is 0.646 (95% confidence interval: 0.546-0.745); McNemar's test p-value is <0.001

^c Excludes *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Malassezia globosa*, *Malassezia nana*, *Malassezia restricta*, *Trichosporon asahii*, and *Yarrowia lipolytica*.

^d false positive is *Alternaria* spp.

Table 13. Comparison of PCR-based Assay Versus Angioinvasion for Identification of Fungus in Formalin-fixed Paraffin-embedded Tissue^a

<u>PCR assay</u>	<u>Histopathology</u>		Total
	Negative for Angioinvasion (Controls)	Positive for Angioinvasion	
Negative for fungal DNA	117	8	125
Positive for fungal DNA^b	1 ^c	40	41
Total	118	48	166

^a Kappa coefficient for comparison is 0.862 (95% confidence interval: 0.775-0.949); McNemar's test p-value is 0.020

^b Excludes *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Malassezia globosa*, *Malassezia nana*, *Malassezia restricta*, *Trichosporon asahii*, and *Yarrowia lipolytica*.

^c false positive is *Alternaria* spp.

Table 14. PCR-Based Assay Test Performance Compared to Histopathology

	PCR Results, % (95% confidence interval)	
	Specimens from Sites with Any Histopathology^a (N=95)	Specimens from Sites with Angioinvasion (N=48)
Sensitivity	63.2 (52.6-72.8)	83.3 (69.8-92.5)
Specificity	99.2 (95.4-99.9)	99.2 (95.4-99.9)
Positive Predictive Value	98.4 (89.4-99.8)	97.6 (85.0-99.7)
Negative Predictive Value	77.0 (72.0-81.3)	93.6 (88.6-96.5)
False Positive Rate	0.8%	0.8%
False Negative Rate	36.8%	16.7%
Positive Likelihood Ratio	75.8	98.3
Negative Likelihood Ratio	0.37	0.17

^a Includes both angioinvasion and nonvascular tissue invasion

Table 15. Frequency of Fungi from Sites with PCR False Negative (Histopathology Confirmed) Findings^a

Culture Findings, No. (%)	Sites with Angioinvasion (N=8)	Sites with Nonvascular tissue invasion (N=27)
Cultures sent for analysis	7	23
No fungi	1 (14.3)	10 (43.5)
Fungal growth	6 (85.7)	13 (56.5)
Order Mucorales	4 (57.1)	5 (21.7)
<i>Saksenaea</i> spp.	1 (14.3)	0
<i>Rhizopus</i> spp.	1 (14.3)	1 (4.3)
<i>Mucor</i> spp.	2 (28.6)	4 (17.4)
<i>Mucor circinelloides</i>	1 (14.3)	0
<i>Aspergillus</i> spp.	2 (28.6)	4 (17.4)
<i>A. terreus</i>	0	1 (4.3)
<i>A. flavus</i>	1 (14.3)	2 (8.7)
<i>A. spp</i>	0	2 (8.7)
<i>A. fumigatus</i>	1 (14.3)	0
<i>Fusarium</i> spp.	3 (42.6)	2 (8.7)
Combinations		
Mucorales plus <i>Aspergillus</i> spp.	1 (14.3)	0
<i>Aspergillus</i> plus <i>Fusarium</i> spp.	0	0
Mucorales plus <i>Fusarium</i> spp.	2 (28.6)	0

^a Specimens with false negative findings were collected from 2009-2013 with the majority between 2011-2012

Table 16. Quality Assurance Review of Specimens^a

Fungal Positivity Findings, No. (%)	UTHSCSA Result ^b	LADR Result ^b	Kappa Coefficient
Specimens sent to both laboratories	62	62	
No fungi	15 (24.2)	6 (9.7)	0.171 (-0.090, 0.432)
Any fungi	47 (75.8)	56 (90.3)	
Fungi identified at genus level	47 (75.8)	56 (90.3)	0.171 (-0.090, 0.432)
Order Mucorales	29 (46.8)	41 (66.1)	0.494 (0.297, 0.692)
<i>Saksenaea</i> spp. ^c	21 (33.9)	15 (24.2)	0.458 (0.222, 0.694)
<i>Aspergillus</i> spp.	15 (24.2)	28 (45.2)	0.423 (0.214, 0.631)
<i>Fusarium</i> spp.	5 (8.1)	8 (12.9)	0.402 (0.046, 0.759)

UTHSCSA - University of Texas Health Sciences Center San Antonio, Texas

^a Data are on a specimen level and not injury site level

^b Five controls specimens were also analyzed. Four were negative for both laboratories. One control specimen had a false positive for *Aspergillus* spp. at LADR, while being negative at UTHSCSA.

^c Specimens identified as *Apophysomyces elegans* were included with *Saksenaea* spp. as LADR was unable to differentiate between the two organisms

Table 17. Quality Assurance Review of Specimens from sites with Angioinvasion^a

Fungal Positivity Findings, No. (%)	UTHSCSA Result ^b	LADR Result ^b	Kappa Coefficient
Specimens sent to both laboratories	37	37	
No fungi	8 (21.6)	1 (2.7)	0.183 (-0.131, 0.497)
Any fungi	29 (78.4)	36 (97.3)	
Fungi identified at genus level	29 (78.4)	36 (97.3)	
Order Mucorales	22 (59.5)	31 (83.8)	0.318 (0.044, 0.593)
<i>Saksenaea</i> spp. ^b	17 (46.0)	13 (35.1)	0.446 (0.161, 0.731)
<i>Aspergillus</i> spp.	8 (21.6)	17 (46.0)	0.377 (0.113, 0.641)
<i>Fusarium</i> spp.	2 (5.4)	5 (13.5)	0.226 (-0.218, 0.670)

UTHSCSA - University of Texas Health Sciences Center San Antonio, Texas

^a Data are on a specimen level and not injury site level

^b Specimens identified as *Apophysomyces elegans* were included with *Saksenaea* spp. as LADR was unable to differentiate between the two organisms

Table 18. Adherence to Joint Trauma System Guidelines for Collection of Tissue Specimens in Blast Trauma Patients (February 2011 – December 2014)^a

	LRMC			U.S. hospitals		
	Total	Specimen Collection, No. (%)		Total	Specimen Collection, No. (%)	
		Histopathology	Fungal Culture		Histopathology	Fungal Culture
<i>Number of criteria met for IFI Risk^b</i>						
0	803	13 (1.6)	15 (1.9)	300	14 (4.7)	25 (8.3)
1	650	42 (6.5)	45 (6.9)	354	49 (13.8)	56 (15.8)
2	249	44 (17.7)	48 (19.3)	174	40 (23.0)	54 (31.0)
3	104	67 (64.4)	67 (64.4)	93	57 (61.3)	66 (71.0)
4	94	77 (81.9)	78 (85.5)	87	67 (77.0)	77 (88.5)
Classified as at risk for IFI (≥3 criteria)	198	144 (72.7)	145 (73.2)	180	124 (68.9)	143 (79.4)
<i>Number of conditions met for DCBI^c</i>						
0	225	2 (0.9)	3 (1.3)	62	1 (1.6)	3 (4.8)
1	544	7 (1.3)	6 (1.1)	217	13 (6.0)	19 (8.8)
2	521	14 (2.7)	17 (3.3)	265	26 (9.8)	38 (14.3)
3	331	46 (13.9)	48 (14.5)	217	45 (20.7)	52 (24.0)
4	139	69 (49.6)	74 (53.2)	117	52 (44.4)	63 (53.9)
Classified as DCBI (5 conditions met)	140	104 (74.3)	105 (75.0)	130	90 (69.2)	103 (79.2)

DCBI – Dismounted Complex Blast Injury; IFI – invasive fungal wound infection; LRMC – Landstuhl Regional Medical Center;

^a Adherence is defined by the collection of tissue specimens for histopathology/culture at the respective hospitals from subjects classified as at risk for IFI or DCBI.

^b At risk for IFI criteria for LRMC Blast Protocol are in **Table 2**. For the 2012 JTS CPG,⁷ patients were considered at high risk for IFI if they had at least three IFI risk factors: 1) dismounted blast injury; 2) above knee traumatic amputation (or progressive transition from

below knee to through knee to above knee amputation); 3) extensive perineal, genitourinary, and/or rectal injury; and 4) massive transfusion > 20 units packed red blood cells plus whole blood. The CPG listed >25 units; however, further examination of risk factors identified >20 units as an independent predictor.⁸

^c A DCBI is characterized by an amputation of one lower extremity (at knee or above) with either an amputation or severe injury to the opposite lower extremity, as well as pelvic, abdominal, or urogenital injury.