Development of a Rapid Multiplex Molecular Assay for the Detection of Bacteria from Necrotic Wounds

Cheryl Sesler, MS
cheryl.sesler@diatherix.com
Donna B. Hockman, MS
Leslie L. Malone, MS, MB(ASCP)^{CM}
Don Stalons, PhD, D(ABMM), MPH
Elena Grigorenko, PhD

Diatherix Laboratories, LLC, Huntsville, AL

Abstract

Introduction: Wound infections can be severe and result in surgery, sepsis, or death without rapid diagnosis and treatment. Skin punctures or internal concussions facilitate a unique microenvironment allowing bacteria or toxin migration to the bloodstream. Current diagnostics can take 48 hours or more to accurately identify bacterial organisms and require specific growth conditions. Multiplex screening for bacteria from necrotic wounds is either not available or has poor sensitivity due to suboptimal extraction or amplification. Rapid and sensitive multiplex detection of bacteria from necrotic wounds is necessary to improve outcomes for infected patients.

Methods: Nested primers were designed to amplify gene targets specific for *Bacteroides fragilis*, *Clostridium septicum/novyi*, *Clostridium perfringens*, *Staphylococcus aureus*, *Kingella kingae*, *Streptococcus pyogenes*, *Staphylococcus lugdunensis*, methicillin resistance, and the Panton-Valentine Leukocidin gene. Specificity was verified with SYBR real-time PCR, and all target primers were combined into a Target Enriched Multiplex Polymerase Chain Reaction (TEM-PCR™) Necrosis Panel primer mix. DNA extraction methods were evaluated using *S. pyogenes* (1e2 to 1e5 cfu/mL) and *C. perfringens* (1e4 and 1e1 cfu/mL). TEM-PCR™ was performed, and PCR products were hybridized to probes coupled to barcoded magnetic beads and analyzed on the Applied BioCode-2000. PCR enzymes were evaluated for multiplex PCR amplification efficiency using a selection of panel organisms.

Results: Two of ten extraction methods indicated *S. pyogenes* and *C. perfringens* detection at 1e2 and 1e1 cfu/mL, respectively. One extraction protocol did not isolate detectable levels of organisms. Two of seven PCR enzymes resulted in comparable results with the detection of *S. aureus*, *S. lugdunensis,* and *C. septicum* ≤ 1e3 cfu/mL; *K. kingae* and *S. pyogenes* ≤ 1e2 cfu/mL; and *B. fragilis* and *C. perfringens* ≤ 1e1 cfu/mL. Methicillin resistance and Panton-Valentine Leukocidin genes were detected in *S. aureus* BAA-1556 ≤ 1e3 cfu/mL. One PCR enzyme amplified internal controls but did not amplify panel targets. Lot-to-lot variability testing of the two comparable enzymes yielded similar performance between lots of each enzyme and higher detection signals (41-548%) observed for one enzyme. False positive detection was ≤2.7%.

Conclusions: Process optimization of reagents and protocols improves sensitivity and performance of diagnostic assays, particularly with more efficient extraction and amplification methods. The TEM-PCR™ Necrosis Panel provides sensitive, specific, and rapid results. Accurate and prompt identification of bacteria from necrotic wounds can improve patient outcomes by preventing severe complications, such as amputation, sepsis, or death.

Introduction

- Severe or necrotic wound infections may result in limb amputation, sepsis, or death. Rapid and accurate diagnosis of severe infections is critical to improve patient treatment and prognosis.[1]
- *Streptococcus pyogenes* is the most common bacterial organism found in wound infections.[2] Although rapid antigen tests are available for *S. pyogenes*, sensitivity is low and does not detect other organisms.
- Skin punctures or internal abscesses provide nutrients (blood) and hypoxic conditions.[3]
- A wound infection creates a unique microenvironment favorable for the growth of fastidious organisms (e.g. *Clostridium spp.*, *Bacteroides spp.*, and *Kingella kingae)*, causing severe necrotic infections, internal abscesses, and bone/joint infections.[3,4,5]
- Skin and soft tissue infections can take 48 hours or more to diagnose using routine microbiological techniques.
- Rapid multiplex molecular screening for bacteria from necrotic wounds is either not available or has poor sensitivity partly due to suboptimal sample processing.

Materials & Methods

Assay Design and qPCR Verification. Using the National Center for Biotechnology Information database, nested primers were designed to amplify gene targets described in Table 1. Gene targets for individual primer pairs were amplified using SYBR chemistry. Briefly, 20 µL qPCR reactions were prepared with 4 µL template, 200 nM primers, Multiplex Master Mix (Qiagen, Venlo, Netherlands), and SYBR I Stain (Thermo Fisher Scientific, Waltham, MA). PCR analyses were performed on the ABI StepOne cycler.

Table 1. Target organisms and genes detected by the Diatherix TEM-PCR™ Necrosis Panel.

Target Organism or Gene	Target Acronym
Bacteroides spp.	BAF
Clostridium novyi/septicum	CLOS
Clostridium perfringens	PERF2
Kingella kingae	KIK
Staphylococcus aureus	NUC
Staphylococcus lugdunensis	LUG
Streptococcus pyogenes	SPY3
Methicillin Resistance	MECA
Panton-Valentine leukocidin	PVL

Materials & Methods *(continued)*

Nucleic Acid Extraction. Nucleic acid was extracted from diluted titered Zeptometrix (Buffalo, NY) organisms: *C. perfringens* Type A (1e4 and 1e1 cfu/mL), *S. pyogenes* Z018 (1e5, 1e4, 1e3, and 1e2 cfu/mL), and *Staphylococcus epidermidis* (1e4, 1e3, and 1e2 cfu/mL). Extractions were performed on the KingFisher™ Flex platform (Thermo Fisher Scientific) and utilized buffers and components from Omega BioTek (Norcross, GA), MO BIO Laboratories (Carlsbad, CA), BioMérieux (Durham, NC), Stratec Biomedical (Birkenfeld, Germany), Sigma-Aldrich (St. Louis, MO), Ambi Products (Lawrence, NY), or Thermo Fisher Scientific. Use of proteinase K (40 mg/mL), lysozyme (50 mg/mL), or lysostaphin (1 mg/mL) varied between methods. Sample volume ranged from 200-275 µL. Initially, ten magnetic bead-based extraction methods were evaluated (Table 2, methods 1-10). Subsequent extractions (methods 11-14) were performed to optimize the XP2 Binding Buffer-based procedure (method 2) and were tested in octuplicate.

Table 2. Extraction methods evaluated during the development of the Diatherix Necrosis Panel.

Extraction	Manufacturer Components (Primary)	Magnetic Beads	Lysis Enzymes
1	MB1 Buffer*, MSL Buffer*	SC*	Proteinase K*, Lysozyme§, Lysostaphin#
2	XP2 Binding Buffer*, MBX1 Buffer*	CND*, CNR*	Lysozyme
3	ClearMag Binding†, B1†, ProK Digestion 1	ClearMag†	Proteinase K
4	ClearMag Binding, B1, ProK Digestion 1	ClearMag	Proteinase K, Lysozyme, Lysostaphin
5	ClearMag Binding, B1, ProK Digestion 2	ClearMag	Proteinase K
6	ClearMag Binding, B1, ProK Digestion 2	ClearMag	Proteinase K, Lysozyme, Lysostaphin
7	NucliSENS Lysis Buffer‡	NucliSENS Magnetic silica‡	None
8	NucliSENS Lysis Buffer	NucliSENS Magnetic silica	Lysozyme, Lysostaphin
9	Stratec InviMag Kit‖, Carrier RNA*	Stratec InviMag Kit	Proteinase K, Lysozyme
10	Viral Nucleic Acid Kit‖	Viral Nucleic Acid Kit	Proteinase K
11	XP2 Binding Buffer, 1X TE Buffer	CND, CNR	Lysozyme
12	XP2 Binding Buffer, MBX1 Buffer	CND, CNR	Proteinase K, Lysozyme
13	XP2 Binding Buffer, MBX1 Buffer	SwiftMag†	Lysozyme
14	XP2 Binding Buffer, 1X TE Buffer	SwiftMag	Lysozyme

*Omega BioTek, †MO BIO Laboratories, ‡BioMérieux, §Stratec Biomedical, ‖Thermo Fisher Scientific, ¶Sigma-Aldrich, #Ambi Products

Target Enriched Multiplex Polymerase Chain Reaction (TEM-PCR™) Setup. Isolated DNA from organisms was amplified with Necrosis Panel TEM-PCR™. Briefly, 20 µL reactions were prepared in triplicate using enzymes shown in Table 3 and an optimized Necrosis Panel multiplexed primer mix. The primer mix contains two pairs of gene-specific primers (Fo, forward out; Fi, forward in; Ri, reverse in; Ro, reverse out) for each target. Fi and Ri primers have a unique tag sequence complementary to the superprimers (Fs and Rs), also included in the primer mix (Figure 1). TEM-PCR™ cycling includes initial enrichment and tagging of each target, followed by traditional PCR amplification.

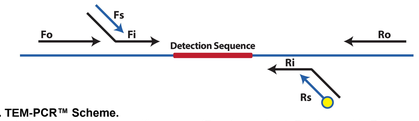

Figure 1. TEM-PCR™ Scheme.
Low concentration nested gene-specific primers (Fo – forward out, Fi – forward in Ri – reverse in, and Ro – reverse out) are designed to enrich the targets during initial PCR cycles. Later in the cycling, a pair of universal superprimers (Fs and Rs) is used to amplify all targets. The Rs primer is labeled with biotin for subsequent detection.

Table 3. Enzymes (or kits) evaluated during Necrosis Panel TEM-PCR™ development.

Enzyme	Enzyme or Kit	Manufacturer	Activation Time (mm:ss)
1	Multiplex Master Mix Kit	Qiagen	15:00
2	Platinum Multiplex Master Mix	Life Technologies	02:00
3	2G Fast Multiplex	KAPA Biosystems	03:00
4	HotStart Taq	New England Biolabs	00:30
5	MyTaq HotStart	Bioline USA	02:00
6	Developmental Master Mix (4)	Enzymatics	03:00
7	SsoAdvanced Universal Probe	BioRad	03:00

Hybridization and Detection. TEM-PCR™ amplicons were hybridized to detection probes coupled onto barcoded magnetic beads (BMBs; Applied BioCode, Santa Fe Springs, CA). Streptavidin-phycoerythrin (SA-PE) was added to the hybridization reaction. After a series of wash steps, fluorescence signals were analyzed on the Applied BioCode-2000 reader.

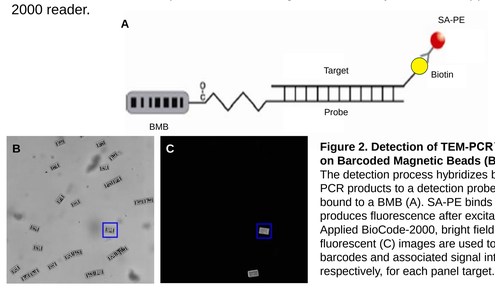

Figure 2. Detection of TEM-PCR™ Amplicons on Barcoded Magnetic Beads (BMBs).
The detection process hybridizes biotinylated PCR products to a detection probe covalently bound to a BMB (A). SA-PE binds biotin and produces fluorescence after excitation. Using the Applied BioCode-2000, bright field (B) and fluorescent (C) images are used to distinguish barcodes and associated signal intensity, respectively, for each panel target.

Results

Initial Primer Verification

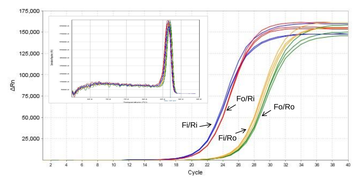

Figure 3. Functionality of target primers was verified prior to multiplexing. Nested primers for each panel target were verified with singleplex SYBR Green qPCR. A representative amplification curve and melt curve (inset) display target amplification and melt peaks for the BAF target primer combination using template DNA isolated from *B. fragilis* Z029.

Extraction Evaluation

Table 4. Detection of *S. pyogenes* and *C. perfringens* target organisms [cfu/mL] during the initial extraction method evaluation for Necrosis Panel development.

Extraction	*S. pyogenes*				*C. perfringens*	
	1e5	1e4	1e3	1e2	1e4	1e1
1	X	X			X	X
2	X	X	X	X	X	X
3	X	X			X	
4	X	X	X		X	
5	X	X			X	
6	X	X	X		X	
7	X	X			X	X
8	X	X	X	X	X	X
9						
10	X				X	

X, detected in at least one replicate

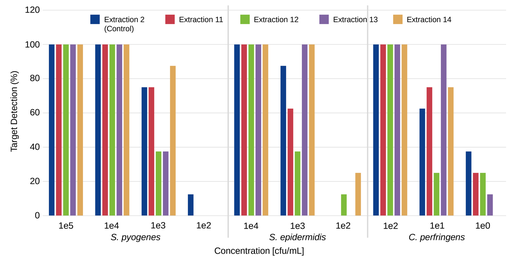

Figure 4. Extraction efficiency varies for representative Necrosis Panel target organisms. Four variations of the initial XP2 Binding Buffer extraction (Table 2, extraction method 2) were examined. In each extraction, *S. pyogenes* was tested from 1e5 to 1e2 cfu/mL, *S. epidermidis* from 1e4 to 1e2 cfu/mL, and *C. perfringens* from 1e4 and 1e1 cfu/mL. TEM-PCR™ was performed using Qiagen Multiplex Master Mix.

TEM-PCR™ Enzyme Evaluation

Table 5. Detection of Necrosis Panel target organisms [cfu/mL] during initial TEM-PCR™ enzyme evaluation.

Enzyme	*C. novyi*		*C. septicum*		MRSA		*S. lugdunensis*		*B. fragilis*		*K. kingae*		*S. pyogenes*	
	1e3	1e2	1e3	1e2	1e4	1e3	1e4	1e3	1e3	1e2	1e3	1e2	1e3	1e2
1	X	X	X	X	X	X	X	X	X	X	X	X	X	
2	X	X	X	X	X	X	X	X	X	X	X	X	X	
3	X	X	X	X	X	X			X	X	X	X	X	
4*														
5	X	X	X	X	X	X	X		X		X	X	X	
6	X				X	X			X		X		X	
7	X	X	X		X	X			X	X	X		X	X

X, detected in at least one replicate; *Internal sample controls were detected with enzyme 4, but no targets were detected.

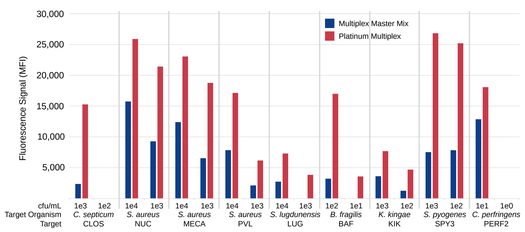

Figure 5. Platinum Multiplex shows increased signals for Necrosis Panel targets compared to Multiplex Master Mix. DNA was isolated from Necrosis Panel target organisms using extraction method 14 (Table 2). Necrosis Panel TEM-PCR™ was performed using three different lots of Multiplex Master Mix and Platinum Multiplex Master Mix, respectively. Median fluorescent intensity (MFI) values were averaged.

Results *(continued)*

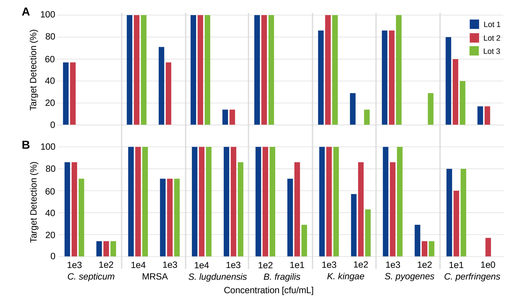

Figure 6. Platinum Multiplex Master Mix has increased sensitivity and lower lot-to-lot variability than Multiplex Master Mix. DNA was isolated from Necrosis Panel target organisms using extraction method 14 (Table 2) in septuplicate. Necrosis Panel TEM-PCR™ was setup using three different lots of Multiplex Master Mix (A) and Platinum Multiplex Master Mix (B).

Discussion

- Efficient cell lysis varies between microorganisms. For the Diatherix Necrosis Panel, a single extraction method must be utilized for all target organisms. *S. epidermidis*, *C. perfringens*, and *S. pyogenes* are gram positive and may be difficult to lyse with standard extraction methods. Due to the sporulation of *C. perfringens* and the hyaluronic acid capsule of *S. pyogenes*, bacterial lysis can be less efficient.[6,7]
- Efficient lysis of target organisms was achieved by combining extraction reagents from multiple vendors.
- TEM-PCR™ is a complex technology with highly multiplexed reactions and unique cycling conditions. Evaluation of enzymes is critical for optimal target sensitivity. Detection of target organisms with Multiplex Master Mix and Platinum Multiplex Master Mix were comparable in the initial enzyme evaluation. Lot-to-lot variability of any reagent is an important factor in molecular diagnostics.
- Increasing target sensitivity is a challenge for any multiplex assay and can result in an increased false positive rate. The rate of false positives for any individual Necrosis Panel target in this developmental dataset was ≤ 2.7%.

Conclusions

- The combination of XP2 Binding Buffer, SwiftMag Beads, and lysozyme provided the most efficient lysis conditions for Necrosis Panel target organisms.
- Platinum Multiplex Master Mix provided the best sensitivity and lot-to-lot performance between PCR enzymes.
- The Diatherix Necrosis Panel will provide accurate and prompt identification of bacteria from necrotic wounds, ultimately improving patient outcomes.

Acknowledgements

We would like to express thanks to Jordan Gallardo, Kelli Grissom, and Jennifer Kelly for contributions to the optimization and development of the Necrosis Panel.

References

[1]Stevens DL, et al. Practice Guidelines for the Diagnosis and Management of Skin and Soft Tissue Infections: 2014 Update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2014. doi: 10.1093/cid/ciu296.
[2]"Necrotizing Fasciitis: A Rare Disease, Especially for the Healthy." Centers for Disease Control and Prevention. Apr 2015. Web. 16 Oct 2015.
[3]Phelps JR, Fagan R, and Pirela-Cruz MA. A Case Study of Negative Pressure Wound Therapy to Manage Acute Necrotizing Fasciitis. *Ostomy Wound Manage*. 2006; 52(3):54-59.
[4]Urschel J. Necrotizing soft tissue infections. *Postgrad Med J*. 1999; 75:645-649.
[5]Dartnell J, Ramachandran M, and Katchburian M. Haematogenous acute and subacute paediatric osteomyelitis: a systematic review of the literature. *J Bone Joint Surg Br*. 2012; 94(5):584-595.
[6]Stevens, D.L., et al. *Manual of Clinical Microbiology*. Ed. James Versalovic. 10th ed. Washington, DC:ASM Press, 2011.
[7]Stollerman GH and Dale JB. The Importance of the Group A *Streptococcus* Capsule in the Pathogenesis of Human Infections: A Historical Perspective. *Clin Infect Dis*. 2008; 46(7):1038-1045.

DIATHERIX® Linking Diagnostics to Therapeutics™

www.diatherix.com

TEM-PCR™ is protected by US 7,851,148 B2 properties of Diatherix Laboratories, LLC.

AMP Poster ID30