Evaluation of Extraction Methods for DNA isolation from Bacteria Associated with Necrosis

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Introduction

- Commensal microorganisms like *Staphylococcus epidermidis* and *Staphylococcus aureus* usually reside on the skin as a part of the normal flora; however, these normally innocuous bacteria can cause severe infection after penetration of the normal epidermal and mucosal barriers.[1]
- *Streptococcus pyogenes*, the causative agent of millions of "strep throat" infections a year, can exist in a carrier state for months without eliciting an immune response. [2]
- *Clostridium perfringens*, commonly found in the gastrointestinal tract of humans and animals, has the ability to form endospores that are resistant to heat, radiation, and toxic chemicals, allowing the organism to persist in food products even after processing and in the environment in soil, sewage, and water. [3,4,5]
- The costs associated with wound infections vary depending on the depth of infection. Estimated costs for deep or invasive infections can exceed $30,000 per case due to longer hospital stays, additional care and wound dressings, outpatient and emergency visits, and possible readmission and/or surgery.[6]
- The spore-forming ability of *C. perfringens* and the polysaccharide capsule that encase *S. pyogenes* cause difficulties in the lysis stage of extraction. [2,3]
- DNA extraction is a critical step for the detection of necrosis organisms. During panel development, the optimization of DNA extraction protocols can affect the speed, accuracy, efficiency, and the sensitivity of the panel.

Material and Methods

Titered Bacterial Organisms. Titered organisms were ordered from Zeptometrix (Buffalo, NY).

- *Streptococcus pyogenes* Z018 [1.68E9 cfu/mL]
- *Staphylococcus epidermidis* MSSE [2.29E9 cfu/mL]
- *Clostridium perfringens* Type A [1.06E8 cfu/mL]

DNA Extraction. DNA was extracted from diluted *S. pyogenes*, *S. epidermidis*, and *C. perfringens* using the MagBind XP2 DNA extraction protocol from Omega Biotek (Norcross, GA) with modifications (Figure 1). Each extraction was performed on a Thermo Scientific, Inc. (Waltham, MA) KingFisher Flex system (Figure 2). Each sample concentration was extracted in replicates of eight for each method evaluated.

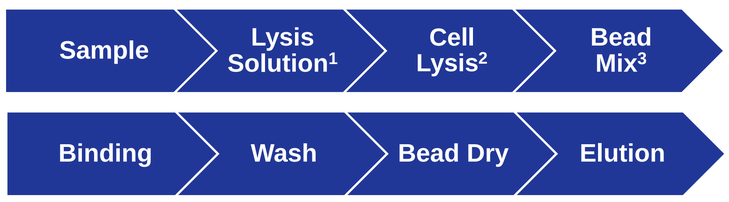

Figure 1. XP2 Extraction Workflow. Modifications for extractions include (1) the use of 1X TE instead of MBX1 buffer in the lysis buffer preparation (Buffer), (2) the addition of Proteinase K during the 20 minute cell lysis step (ProK), and (3) the use of MoBio Laboratories, Inc. (Carlsbad, CA) SwiftMag™ beads in place of the Magbind® particles from Omega Biotek, Inc. (Beads).

Materials and Methods *(Cont'd)*

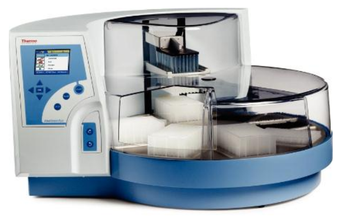

Figure 2. KingFisher Flex system. The KingFisher Flex system from Thermo Scientific, Inc. was used for the semi-automated MagBind XP2 DNA extraction protocol.

Target Enriched Multiplex Polymerase Chain Reaction (TEM-PCR™). Isolated DNA from titered organism dilutions were amplified using the TEM-PCR developmental Gram Positive Necrosis Panel (Table 1). Each PCR reaction contains two pairs of nested gene-specific primers (Fo, forward out; Fi, forward in; Ri, reverse in; Ro, reverse out) for each panel target. Fi and Ri primers are designed with a unique tag sequence that can be recognized by SuperPrimers, also included in the same PCR reaction (Figure 3). An initial target enrichment step occurs prior to typical PCR amplification.

Table 1. Organisms and antibiotic resistance targets detected by the developmental Necrosis Panel.

Organism/Antibiotic Resistance	Target Name
Staphylococcus aureus	nuc4
Staphylococcus spp., coagulase-negative	CoNS
Staphylococcus aureus, methicillin resistant	mecA
Streptococcus pyogenes	SPY3
Panton-Valentine Leukocidin Toxin	pvl
Clostridium perfringens	perf2
Clostridium novyi/septicum	Clos

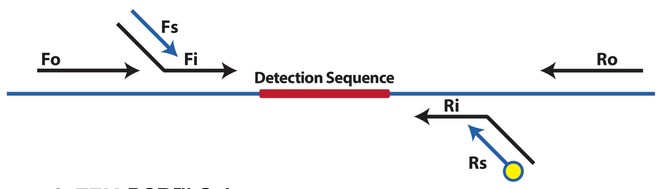

Figure 3. TEM-PCR™ Scheme.
Low concentration nested gene-specific primers are (Fo – forward out; Fi – forward in; Ri – reverse in; and Ro – reverse out) designed to enrich the targets during the initial PCR cycles. Later in the procedure, a pair of universal SuperPrimers (Fs and Rs) is used to amplify all targets. The Rs primer is labeled with biotin for subsequent detection.

Hybridization and Detection. Carboxylated barcoded magnetic beads (BMBs) were coupled to target-specific detection oligonucleotides. Amplified TEM-PCR products were hybridized to coupled bead mixes. Streptavidin-phycoerythrin (SA-PE) was conjugated to biotinylated PCR products. During the hybridization process, excess SA-PE is removed during a series of wash steps. Hybridized products were analyzed on the Applied BioCode 2000 (ABC, Santa Fe Springs, CA).

Figure 4. Hybridization to Coupled BMBs. PCR products bind to the complimentary detection oligonucleotides coupled to the beads. SA-PE is added and binds to the biotin labeled products (A). Once the excess SA-PE is removed, the hybridization plate can be analyzed with the Applied BioCode 2000 (B).

Results

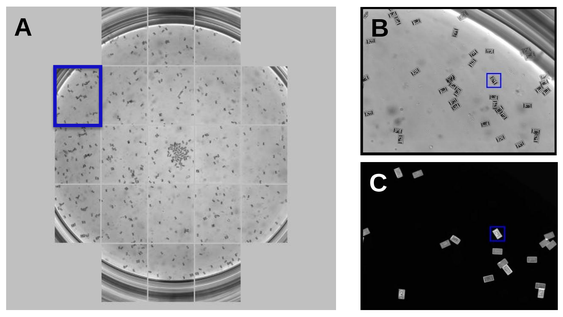

Figure 5. Target detection on the ABC platform. (A) On the ABC Analyzer, a series of images are taken for each well on the hybridization plate. Barcode (target) identification (B) and positive signal (C) are determined form brightfield and fluorescent images, respectively.

Table 2. Detection of *S. pyogenes*, *S. epidermidis*, and *C. perfringens* on the ABC platform using modified XP2 DNA extraction protocols.

Sample (cfu/mL)	Extraction Methods			
	Control	Buffer	ProK	Beads
S. pyogenes 1e5	100% (8/8)	100% (8/8)	100% (8/8)	100% (8/8)
S. pyogenes 1e4	100% (8/8)	100% (8/8)	100% (8/8)	100% (8/8)
S. pyogenes 1e3	75% (6/8)	75% (6/8)	37.5% (3/8)	37.5% (3/8)
S. pyogenes 1e2	12.5% (1/8)	0%	0%	0%
S. epidermidis 1e4	100% (8/8)	100% (8/8)	100% (8/8)	100% (8/8)
S. epidermidis 1e3	87.5% (7/8)	62.5% (5/8)	37.5% (3/8)	100% (8/8)
S. epidermidis 1e2	0%	0%	12.5% (1/8)	0%
C. perfringens 1e2	100% (8/8)	100% (8/8)	100% (8/8)	100% (8/8)
C. perfringens 1e1	62.5% (5/8)	75% (6/8)	25% (2/8)	100% (8/8)
C. perfringens 1e0	37.5% (3/8)	25% (2/8)	25% (2/8)	12.5% (1/8)

Discussion

- The 'Buffer' and control methods each had six positives for *S. pyogenes* at 1e3 cfu/mL, while the other methods detected three positives at the same concentration.
- At 1e3 cfu/mL, all eight *S. epidermidis* samples extracted using the 'Beads' method had positive signal.
- Positive signals were detected in all eight *C. perfringens* samples (1e2 cfu/mL) extracted using the 'Beads' method.

Conclusions

- The 'Beads' method was optimal for the detection of two of the three organisms; however, further studies must be conducted to improve the detection for *S. pyogenes* at lower concentration.
- Future studies will evaluate the combination of the 'Bead' and 'Buffer' extraction methods. Primer mix optimization during TEM-PCR will be conducted to increase sensitivity to all Necrosis organisms.

Acknowledgements/References

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References

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