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Award Number: W81XWH-05-1-0134

TITLE: Rapid Identification of Key Pathogens in Wound Infection by Molecular Means

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REPORT DATE: January 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-01-2006	Annual	15 DEC 2004 - 14 DEC 2005

4. TITLE AND SUBTITLE
Rapid Identification of Key Pathogens in Wound Infection by Molecular Means

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0134

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Brentwood Biomedical Research Institute
Los Angeles, CA 90073

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this study is to develop a real-time PCR-based high throughput detection and identification method for important wound pathogens. This will enable us to provide accurate identification a great deal sooner than has been possible in the past. This is likely to result in improved clinical outcome, less chance of bacterial resistance, and cost savings. To date, we have developed over 20 group- or species-specific primer/probe sets for pathogens encountered in wound infection. The specificities of the primer/probe sets were successfully evaluated with a battery of bacteria. The evaluation of sensitivity is underway. When the entire set of primers and probes has been completed, we will be able to detect, identify and semi-quantitate the important wound pathogens within 8 hours using this TaqMan PCR assay.

15. SUBJECT TERMS
Wound infection, bacteria, molecular identification, real-time PCR

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	21	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Wound infection remains an important problem in both military and civilian life despite availability of a wide array of antimicrobial agents and improvements in surgical technique. Wound infection leads to morbidity and, at times, mortality. Studies have shown that infection of post-surgical wounds adds 10 days to the period of hospitalization and approximately $4,000 to the cost [1,2]. Severe trauma and foreign bodies add to the difficulties in management of military wounds and some civilian wounds. Other problems in wound management relate to the increasing numbers of immunosuppressed patients, the use of extensive surgical procedures in certain situations, and the increasing incidence of microorganisms resistant to antimicrobial agents.

Most wound infections involve both aerobic or facultative bacteria and anaerobic or microaerophilic bacteria. The latter category of organisms often have fastidious growth requirements and may therefore be readily overlooked. The difficulties and time required for good anaerobic bacteriology, along with decreases in available funding, have led many laboratories to do abbreviated workups for anaerobes or to eliminate anaerobic bacteriology entirely. As few as 50 to 75% of anaerobes from various sources are characterized satisfactorily, according to a recent survey [3] and 27% of hospital microbiology laboratories indicated that they never identified anaerobes. Furthermore, virtually all laboratories depend on phenotypic testing alone for identification, typically using commercial kits. These conventional identification protocols are not only laborious and time-consuming, but also often result in inconclusive or even inaccurate identification. In addition, they are culture-dependent and thus more time-consuming. Anaerobic bacteria grow more slowly, but more importantly it takes considerable time to get the various elements of a mixed infection (commonly involving 4 to 12 bacteria) isolated in pure culture so that identification and antimicrobial susceptibility testing can proceed. These delays and limitations can lead to inappropriate antibiotic use in wound infection patients or use of unnecessarily broad-spectrum agents, which contributes to increasing rates of antibiotic resistance. If empirical treatment is ineffective, the patient may deteriorate. The development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases [4]. Rapid diagnosis can be achieved by the direct detection of characteristic bacterial genes in clinical specimens. In recent years, real-time PCR has been applied successfully in the medical field, for example in the quantitation of various DNA and RNA viruses in patients [5-9], in the detection of gene amplification [10], gene mutations [11,12], or chromosomal rearrangements, in the quantitation of gene expression [10,12], and in quantitation of particular pathogens in clinical specimens [13-19]. Here, we describe the use of real-time PCR (TaqMan) assay for high throughput detection of wound infection pathogens.

BODY

[1]: Key pathogens involved in wound infections:

Anaerobes

Gram-positive cocci
Peptostreptococcus micros, P. asaccharolyticus, P. anaerobius, Finegoldia magna

Gram-negative rods:
Prevotella spp., *P. melaninogenicus, P. nigrescens, P. bivia, P. disiens*
Fusobacterium spp., *F. nucleatum, F. mortiferum*
Bacteroides fragilis group: *Bacteroides fragilis, B. thetaiotaomicron, B. vulgatus, B. ovatus, B. uniformis, B. distasonis*
Bacteroides gracilis
Bilophila wadsworthia
Porphyromonas macacae

Gram-positive rods:
Actinomyces odontolyticus, A. viscosus, A. naeslundii, A. israelii
Clostridium perfringens, C. ramosum, C. sordellii, C. novyi
C. clostridioforme group: *C. bolteae, C. clostridoforme*, and *Clostridium hathewayi*

Aerobes

Gram-positive cocci:
Streptococci:
Group A streptococci (*Streptococcus pyogenes*)
Group B streptococci (*Streptococcus agalactiae*)
Streptococcus anginosus group: *S. anginosus, S. constellatus* subsp. *constellatus, S. intermedius*
Staphylococci:
Staphylococcus spp., *S. aureus, S. epidermidis*
MRSA (Methicillin Resistant *Staphylococcus aureus*)
MSSA (Methicillin Sensitive *Staphylococcus aureus*)
PVL toxin (Panton Valentine Leukocidin toxin)
Enterococci:
Enterococcus faecium, E. faecalis

Gram-negative rods:
Enterobacter cloacae, E. aerogenes
Escherichia coli
Klebsiella oxytoca, K. pneumoniae
Pseudomonas aeruginosa

[2] TaqMan probe technology:

The Taqman probe technology combines the features of rapid PCR and real-time detection of an amplification product. It uses the 5'-nuclease assay and FRET (fluorescent resonance energy transfer). The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the *Taq* polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

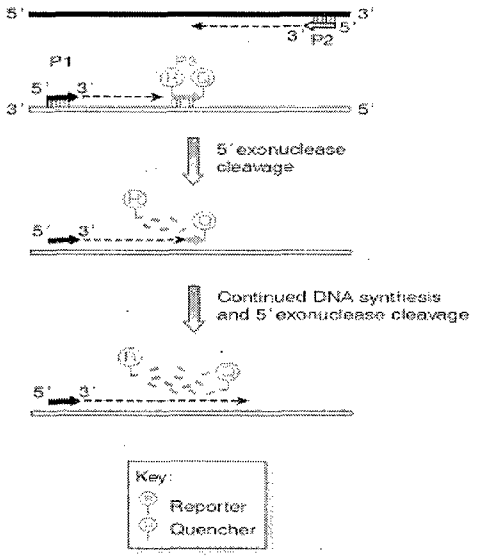

Fig.1. In addition to two conventional PCR primers, P1 and P2, which are specific for the target sequence, a third primer, P3, is designed to bind specifically to a site on the target sequence downstream of the P1 binding site. P3 is labelled with two fluorophores, a reporter dye (R) is attached at the 5' end, and a quencher dye (D), which has a different emission wavelength to the reporter dye, is attached at its 3' end. Because its 3'end is blocked, primer P3 cannot by itself prime any new DNA synthesis. During the PCR reaction, *Taq* DNA polymerase synthesizes a new DNA strand primed by P1 and as the enzyme approaches P3, its 5' –3' exonuclease activity progressively degrades the P3 primer from its 5'end. The end result is that the nascent DNA strand extends beyond the P3 binding site and the reporter and quencher dyes are no longer bound to the same molecule. As the reporter dye is no longer in close proximity to the quencher, the resulting increase in reporter emission intensity is easily detected

Real-time PCR has several advantages over conventional PCR. It focuses on the logarithmic phase of product accumulation rather than on the end-product abundance. Therefore, it is more accurate since it is less affected by amplification efficiency or depletion of a reagent. In addition, it has an increased dynamic range for quantification of target sequence (at least 5 orders of magnitude). Furthermore, without any post-PCR manipulation of the samples, cross-contamination between samples is greatly reduced. Finally, PCR results can be obtained within 2 hours.

(1). ABI PRISM® 7500 Sequence Detection System

ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif.) was purchased, set up and standardized. This system integrates a PCR-based assay with laser scanning technology to excite fluorescent dyes present in the specially designed TaqMan® probes. It is a fully integrated system for real-time detection of PCR. The system includes a built-in thermal cycler, a laser to induce fluorescence, CCD (charge-coupled device) detector, real-time sequence detection software, and TaqMan® reagents for the fluorogenic 5' nuclease assay. All the personnel involved in this project were trained.

(2). TaqMan Primer and Probe design:

Generally, putative genus- or species-specific primer and probe sets were chosen by examining the alignment of the target sequences with sequences of similar organisms

and selecting sequences unique to the organism(s) in question. Sequence alignment was done by using CLUSTAL-W [20] (http://genome.kribb.re.kr). Regions that are unique to sequences representing different genera or species were selected for primer and probe design. All primer and probe sets were subjected to the guidelines established by Applied Biosystems by using Primer Express 2.0 (Applied Biosystems, Foster City, Calif.) The primer and probe sequences were analyzed for T_m (melting temperature), secondary structure formation, G+C content, and primer-dimer formation with the NetPrimer analysis software http://www.premierbiosoft.com/netprimer). We compared potential candidates for PCR primers and probes to the aligned SSU_rRNA database of the Ribosomal Database Project using the CHECK_PROBE utility [21] and to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) [22]. All the primers and probes designed to date are summarized in **Table 1**.

16S rRNA gene:

The genus- or species-specific primer and probe sets finished to date in this study were designed from the 16S rRNA gene for all the following bacteria:

Bacteroides fragilis group species: *B. fragilis*, *B. stercoris*, *B. vulgatus* and *B. thetaiotaomicron*
Bilophila wadsworthia
Prevotella species including *P. disiens*, *P. bivia*, *P. melaninogenica*, and *P. nigrescens*
Porphyromonas macacae (& *Porphyromonas salivosa*)
Gram-positive anaerobic cocci (GPAC) including *Peptostreptococcus anaerobius*, *P. micros* and *Finegoldia magna*
Actinomyces odontolyticus
Pseudomonas aeruginosa

***Spy* 1258 gene:**

A *S. pyogenes*-specific gene (*Spy* 1258) that encodes a putative transcriptional regulator [23] was used for *Streptococcus pyogenes* (Group A streptococcus) specific-specific primer and probe design. It was noted that a stretch of nucleotides identical to *Spy* 1258 (nt. 6651-7193) was also found in the complete genome of S. pyogenes M3 strains MGAS315 (GenBank accession No. AE014154) and SSI-1 (GenBak accession No. AP005144) as well as M18 strain MGAS8232 (GenBank accession No. AE010045). However, the Spy 1258 gene sequence was clearly absent in other bacterial genomes available at GenBank. The primer and probe set was subjected to the guidelines established by Applied Biosystems (Foster City, Calif.) by using Primer Express software (V.2.0).

***sip* gene:**

The primers and probes specific for group B streptococcus (GBS)-*Streptococcus agalactiae* were designed using the *sip* gene [24] which encodes Sip-surface immunogenic protein as this protein is universally expressed in GBS. *S. agalactiae*-

specific primers and probe were designed using Primer Express 2.0 from Applied Biosystems.

***pbp2b* gene:**

The partial penicillin-binding protein 2B gene (*pbp2b* gene) [25] was used for the anginosus group streptococci (AGS)(formerly called "*Streptococcus milleri*")-specific primer and probe design. The primers and probes were designed according to the alignment analysis of *pbp2b* gene sequences of *Streptococcus anginosus* strain MAS624 (GenBank Accession No. AY289802), *Streptococcus intermedius* strain ATCC 27335 (AY289801), *Streptococcus constellatus* subsp. *pharyngis* strain MM9889a (AY289800), *Streptococcus anginosus* strain ATCC 33397 (AY289798), *Streptococcus constellatus* subsp. *constellatus* strain ATCC 27823 (AY289799) with the homologous genes of *Streptococcus mitis* (Z22182), *Streptococcus pneumoniae (*Z22184), *Streptococcus mutans (AE014903)* and *Streptococcus agalactiae* (AL766847).

***tuf* gene:**

The *tuf* gene encodes the elongation factor Tu, which is an essential constituent of the bacterial genome [26]. The *tuf* gene sequences of *Staphylococcus* species (*S. aureus* [AF298796], *S. epidermidis* [AF298800], *S. saprophyticus* [AF298804], *S. haemolyticus* [AF298801], *S. hominis* [AF298802], *S. lugdunensis* [AF298803], *S. simulans* [AF208805], *S. warneri* [AF298806], *S. xylosus* [AY763438], *S. sciuri* [AY763434], *S. cohnii* subsp. *ureolyticus* [AY298799] and *S. capitis* [AF298798]) that were available from GenBank were analyzed by the software mentioned above.

***nuc* gene:**

S. aureus strains produce an extracellular thermostable nuclease (TNase) with a frequency similar to that at which they produce coagulase. Tnase protein has been well characterized and its gene, the *nuc* gene, has been cloned and sequenced [27]. The *nuc* gene is unique to *S. aureus* and it was used for primer and probe design.

(3). TaqMan real-time PCR:

Real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif.). After primers and probes were designed, they were synthesized by Sigma-genosys. Probes were labeled with the reporter dye 6-carboxyfluorescein (6'-FAM) at the 5' end and with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. TaqMan real-time PCR was performed with 3 μl of DNA, 12.5 μl of 2X TaqMan PCR master mix (Applied Biosystems, Foster City, Calif.).), 900 nmol of each primer, and a 200-nmol probe in a final volume of 25 μl. Thermal cycling conditions were as follows: 2 min at 50°C, 10min at 95°C followed by 45 repeats of 15 s at 95°C, and 1min at 60°C. Data collection was performed during each annealing phase.

Table 1. Primers and probes included in this study

Target organisum	Sequence of primer or probe genes (5' end labeled with FAM-6 and 3' end labeled with TRAMA)	Target	Tm	GC%	Length	Reference
**B. fragilis*	Bfrag-F: 5'-GCGGGTGACCGTATGCTAAT-3'	16s	60.0	55.0	20	This
	Bfrag-R: 5'-TCACGAAGTCGGGTTGCA-3'	rRNA	58.4	55.5	18	study
	Bfrag-P: 5'-CCAAAATCCTCTCTCAGTTCGGATCGAAG-3'		71.5	48.2	29	
**B. thetaiotaomicron*	Bthe-F: 5'-CGGGCTTAAATTGCATTTG-3'	16s	57.1	42.1	19	This
	Bthe-R: 5'- CCATGCAGCACCTTCACATT-3'	rRNA	59.0	50.0	20	study
	Bthe-P: 5'-ATAATCTGGAAACAGGTTAGCCGCAAGG-3'		70.0	46.4	28	
** B. stercoris*	Bster-F: 5'-TTGCAACTGACTGAATCGGAA-3'	16s	59.0	42.8	21	This
	Bster-R: 5'-CTGACGACAACCATGCAGCA-3'	rRNA	60.9	55.0	20	study
	Bster-P: 5'-CGGTTCTTTCCTTCGGACAGTTGTGAA-3'		70.6	48.1	27	
**B. vulgatus*	Bvul-F: 5'-CGGGCTTAAATTGCAGATGA-3'	16s	58.9	45.0	20	This
	Bvul-R: 5'-CATGCAGCACCTTCACAGAT-3'	rRNA	57.4	50.0	20	study
	Bvul-P: 5'-GGTGAAAGCCGTAAGCCGCAAGG-3'		70.8	60.8	23	
**Bilophila wadsworthia*	Bwads-F: 5'-GGCTGGAAACGGTCGCTAA-3'	16s	61.0	57.8	19	This
	Bwads-R: 5'-GGACTCATCCTTAAGCGATAGC-3'	rRNA	57.9	50.0	22	study
	Bwads-P: 5'-CGAATACGCTCCCGATTTTATCATTGGG-3'		72.2	46.4	28	
Prevotella spp.	Prev-F: 5'-CCAGCCAAGTAGCGTGCA-3'	16s	58.6	61.1	18	[28]
	Prev-R: 5'-TGGACCTTCCGTATTACCGC-3'	rRNA	60.0	55.0	20	
	Prev-P: 5'-CAAATCTGATGCCGTCATCGAAGACTATGC-3'		72.7	46.7	30	
**P. nigrescens*	Pnig-F: 5'-GGCCTAATACCCGATGTGTTT-3'	16s	58.5	47.6	21	This
	Pnig-R: 5'-ACGCATCCCCATCCCTTA-3'	rRNA	58.1	55.5	18	study
	Pnig-P: 5'TGACGGCATCCGATATGAAACAAAGG-3'		70.0	46.1	26	
Pseudomonas aeruginosa	Pseu-F: 5'- TGCGATCACCACCTTCTACTT-3'	16s	58.9	47.6	21	This
	Pseu-R: 5'- TTTCGCGTGTACGTCCAGA-3'	rRNA	59.0	52.6	19	study
	Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3'		71.2	61.5	26	

Target organisum	Sequence of primer or probe genes (5' end labeled with FAM-6 and 3' end labeled with TRAMA)	Target	Tm	GC%	Length	Reference
**P. disiens*	Pdis-F: 5'-GCCGGTACAGAATGTTGGTT-3'	16S	57.4	50.0	20	present
	Pdis-R: 5'-GGTTCGTGAGGTCGGGTT-3'	rRNA	57.6	61.1	18	study
	Pdis-P: 5'-AATGTAATCTAATCTTTAAAGCCGGTCCCAGTTC-3'		71.5	38.2	34	
**P. bivia*	Pbiv-F: 5'-GGTGGTACAGATAGTTGGTCGTG-3'	16S	58.8	52.1	23	present
	Pbiv-R: 5'-TTGCAGACCCCAGTCCGA-3'	rRNA	59.9	61.1	18	study
	Pbiv-P: 5'-GCAAATACGATCTAATCCTTAAAACCATTCTCAG-3'		69.5	35.3	34	
P. melaninogenica	Pmel-F: 5'-GTGGGATAACCTGCCGAAAG-3'	16S	59.6	55.0	20	[28]
	Pmel-R: 5'-CCCATCCATTACCGATAAATCTTTA-3'	rRNA	61.2	36.0	25	
	Pmel-P: 5'-GCATAGTCTTCGATGACGGCATCAGATTTG-3'		72.7	46.6	30	
**Porphyromonas macacae*	Pmac-F: 5'-TTGAAATTTAGCGGACTATGTATG-3'	16S	56.8	33.3	24	present
	Pmac-R: 5'-AGCTGACGACAACCATGCA-3'	rRNA	57.6	52.6	19	study
	Pmac-P: 5'-TACATATCCTGTCACAAGGCCGCTAAGTAGG-3'		70.9	48.3	31	
Fusobacterium spp.	Fuso-F: 5'-AAGCGCGTCTAGGTGGTTATGT-3'	16S	65.8	50.0	22	[28]
	Fuso-R: 5'-TGTAGTTCCGCTTACCTCTCCAG-3'	rRNA	60.6	52.1	23	
	Fuso-P: 5'-CAACGCAATACAGAGTTGAGCCCTGCATT-3'		73.1	48.2	29	
**Finegoldia magna*	Fmag-F: 5'-AGGGTACGCAGGCGGTTTAAT-3'	16S	62.8	52.3	21	present
	Fmag-R: 5'-TCAGTTTCCAATGCTTTACGG-3'	rRNA	58.2	42.8	21	study
	Fmag-P: 5'-AGTCGAATGTTAAAGATCGGGGCTCAAC-3'		70.0	46.4	28	
**Micromonas micros*	Pmic-F: 5'- GCCGTTGGAAACGACGATTA-3'	16S	61.0	50.0	20	present
	Pmic-R: 5'- CACCGATAAATCTTTGACCCCTAT-3'	rRNA	60.9	41.6	24	study
	Pmic-P: 5'-TACCGCATGAGACCACAGAATCGCA-3'		70.4	52.0	25	
**P. anaerobius*	Pana-F: 5'-GGCGTAAAGGGTGCGTAGGT-3'	16S	61.7	60.0	20	present
	Pana-R: 5'-TTCGGAGGCTAACTACGGTTGA-3'	rRNA	61.6	50.0	24	study
	Pana-P: 5'-TCTTTCAAGTCGGTGGTTAAAGGCTACGG-3'		71.6	48.2	29	
**Actinomyces odontolyticus (& A. lingnae)*	Aodo-F: 5'-ATGTGGTGGCATTTAGTTGGTC-3'	16S	59.0	45.4	22	present
	Aodo-R: 5'-GACTTAACCCAACATCTCACGAC-3'	rRNA	58.1	47.8	23	study
	Aodo-P: 5'-GGTGGTGCATGGTTGTCGTCAGCTCG-3'		75.4	61.5	26	

Clostridium perfringens	CPerF165F: 5'-CGCATAACGTTGAAAGATGG-3'	16S	56.8	45.0	20	[29]
	CPerf269R: 5'-CCTTGGTAGGCCGTTACCC-3'	rRNA	59.4	63.1	19	
	Cperf-P: 5'-TCATCATTCAACCAAAGGAGCAATCC-3'		67.5	42.3	26	
**Staphylococcus* spp.	Staph-F: 5'-CAAC(T/A)CCAGAACGTGAT(T/C)CTG-3'	*tuf*	58.6	52.6	21	present
	Staph-R: 5'-CAGTACCACGACCAGTGATTGAG-3'	gene	59.6	52.1	23	study
	Staph-P: 5'-AAACCATTCATGATGCCAGTTGAGGACG-3'		72.0	46.4	28	
* *S. aureus*	Saur-F: 5'- AGCTCAGCAAATGCATCACAA-3'	*nuc*	59.2	42.8	21	present
	Saur-R: 5'-TTAGTTGAAGTTGCACTATATACTGTTGGAT-3'	gene	62.6	32.2	31	study
	Saur-P: 5'-CAGATAA(C/T)GGCGTAAATAGAAGTGGTTC- 3'		67.5	40.7	32	
S. epidermidis	Sepi-F: 5'-TACACACCGCCCGTCACA-3'	16S	59.0	61.0	18	[30]
	Sepi-R: 5'-CTTCGACGGGCTAGCTCCAAAT-3'	rRNA	64.7	54.5	22	
	Sepi-P: 5'-CACCCGAAGCCGGTGGAGTAACC-3'		70.5	65.2	23	
MRSA	MecA-F: 5'-GGCAATATTACCGCACCTCA-3'	*Mec*A	58.6	50.0	20	[31]
	MecA-R: 5'-GTCTGCCACTTTCTCCTTGT-3'	gene	53.9	50.0	20	
	MecA-P: 5'-AFATCTTATGCAAACTTAATTGGCAAATCC-3'		69.0	32.0	30	
PVL	PVL-F: 5'-ACACACTATGGCAATAGTTATTT-3'	PVL	51.4	31.4	23	[31]
	PVL-R: 5'-AAAGCAATGCAATTGATGTA-3'	gene	51.8	30.0	20	
	PVL-P: 5'-ATTTGTAAACAGAAATTACACAGTTAAATATGA-3'		61.67	21.0	33	
*Group A *Streptococcus*	Spyo-F: 5'-CAAAAATGACACTCTGGATGATTTG-3'	*Spy*	60.7	36.0	25	present
	Spyo-R: 5'-GGACAAGGTTTGATTGAGGACTTG-3'	1258	61.9	45.8	24	study
	Spyo-P: 5'-CCGTTTGTTAAATCAGGCTGAAATCTACACAGAC-3'		72.1	41.1	34	
*Group B *Streptococcus*	GBS-F: 5'-TCAGTCGCAAGTGTTCAAGCA-3'	*Sip*	60.0	47.6	21	present
	GBS-F: 5'-AAATCAGCCTTTACCTCTGAAACAG-3'	gene	60.0	40.0	25	study
	GBS-P: 5'-AAGAAACAGATACGACGTGGACAGCACGT-3'		71.2	48.2	29	
*Anginosus group streptococci	SAG-F: 5'-CGGTAGCTAATGG(T/C)GGTACTCGTA-3'	*Pbp2b*	64.0	50.0	28	present
	SAG-R: 5'-ATTTTCCAAG(A/C)CC(A/G)CC(T/C)(T/G)T(T/C)TCAT-3'	gene	64.4	33.3	24	study
	SAG-P: 5'-GCACC(A/G)CACTTAGT(G/A)GAAGGAATTTATGACAA-3'		74.9	42.4	33	

* Primers and probes were designed in present study.

(4). Specificity evaluation for TaqMan PCR assay:

The specificities of primer/probe sets designed in this study were predicted by comparison to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility [21] and to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) [22], and further confirmed by testing against a panel of phylogenetically related bacterial strains. The specificities of the rest of the primer/probe sets have previously been demonstrated [28-32].

Gram-negative anaerobes:

The specificities of the primer and probe sets for *Bacteroides fragilis, B. thetaiotaomicron, B. stercoris, B. vulgatus, Bilophila wadsworthia, Prevotella nigrescens, P. disiens, P. bivia* and *Porphyromonas macacae* were tested against a panel of genomic DNA preparations (Table 2 & 3) from type strains and well-characterized clinical isolates that are phylogenetically related to these species. The primer/probe sets detected only corresponding strain(s), except for that of *B. ovatus* which showed a significant increase in fluorescence indicating cross-reactivity with *B. thetaiotaomicron* species-specific primer and probe. No signal was detected for any other non-corresponding strains.

Table 2. Bacterial strains tested by TaqMan assay

Species	Strains	No. of Strains	Bthe-F/R/P	Bfrag-F/R/P	Bster-F/R/P	Bvul-F/R/P	Pnig-F/R/P	Pdis-F/R/P	Pbiv-F/R/P
Bacteroides thetaiotaomicron	ATCC 29148^T	1	+	-	-	-	NT	NT	NT
Bacteroides thetaiotaomicron	Clinical isolates	6	+	-	-	-	NT	NT	NT
Bacteroides ovatus	ATCC 8483^T	1	+	-	-	-	NT	NT	NT
Bacteroides ovatus	Clinical isolates	6	+	-	-	-	NT	NT	NT
Bacteroides fragilis	ATCC 25285^T	1	-	+	-	-	NT	NT	NT
Bacteroides fragilis	Clinical isolates	6	-	+	-	-	NT	NT	NT
Bacteroides stercoris	ATCC 43183^T	1	-	-	+	-	NT	NT	NT
Bacteroides stercoris	Clinical isolates	6	-	-	+	-	NT	NT	NT
Bacteroides vulgatus	ATCC 8482^T	1	-	-	-	+	NT	NT	NT
Bacteroides vulgatus	Clinical isolates	6	-	-	-	+	NT	NT	NT
Bacteroides distasonis	ATCC 8503^T	1	-	-	-	-	NT	NT	NT
Bacteroides merdae	ATCC 43184^T	1	-	-	-	-	NT	NT	NT
Bacteroides caccae	ATCC 43185^T	1	-	-	-	-	NT	NT	NT
Bacteroides uniformis	ATCC 8492^T	1	-	-	-	-	NT	NT	NT
Bacteroides eggerthii	ATCC 27754^T	1	-	-	-	-	NT	NT	NT
Bacteroide goldsteinii	ATCC BAA- 1180^T	1	-	-	-	-	NT	NT	NT

Table 2 (Cont.)

Bacteroides nordii	ATCC BAA- 998^T	1	-	-	-	-	NT	NT	NT
Bacteroides salyersiae	ATCC BAA-997^T	1	-	-	-	-	NT	NT	NT
Prevotella nigrescens	ATCC 33563^T	1	NT	NT	NT	NT	+		-
Prevotella nigrescens	Clinical isolates	5	NT	NT	NT	NT	+		-
Prevotella disiens	ATCC 29426^T	1	NT	NT	NT	NT	-	+	-
Prevotella disiens	Clinical isolates	5	NT	NT	NT	NT	-	+	-
Prevotella bivia	ATCC 29303^T	1	NT	NT	NT	NT	-	-	+
Prevotella bivia	Clinical isolates	3	NT	NT	NT	NT	-	-	+
Prevotella melaninogenica	ATCC 25845^T	1	NT	NT	NT	NT	-	-	-
Prevotella buccae	ATCC 33574^T	1	NT	NT	NT	NT	-	-	-
Prevotella buccalis	ATCC 35310^T	1	NT	NT	NT	NT	-	-	-
Prevotella corporis	ATCC 33547^T	1	NT	NT	NT	NT	-	-	-
Prevotella denticola	ATCC 33185	1	NT	NT	NT	NT	-	-	-
Prevotella intermedia	ATCC 25611^T	1	NT	NT	NT	NT	-	-	-
Prevotella loescheii	ATCC 15930^T	1	NT	NT	NT	NT	-	-	-
Prevotella oralis	ATCC 33269^T	1	NT	NT	NT	NT	-	-	-
Prevotella oulorum	ATCC 43324^T	1	NT	NT	NT	NT	-	-	-
Prevotella veroralis	ATCC 33779^T	1	NT	NT	NT	NT	-	-	-
Prevotella zoogleoformans	ATCC 33285^T	1	NT	NT	NT	NT	-	-	-
Porphyromonas gingivalis	ATCC 33277^T	1	NT	NT	NT	NT	-	-	-
Porphyromonas endodontalis	ATCC 35406^T	1	NT	NT	NT	NT	-	-	-

Table 3. Bacterial strains tested by TaqMan assay

Species	Strains	No. of Strains	Pmac-F/R/P	Bwads-F/R/P
Porphyromonas macacae	ATCC 33141^T	1	+	NT
Porphyromonas asaccharolytica	ATCC 25260^T	1	-	NT
Porphyromonas endodontalis	ATCC 35406^T	1	-	NT
Porphyromonas levii	ATCC 29147^T	1	-	NT
Porphyromonas gingivalis	ATCC 33277^T	1	-	NT

Porphyromonas gingivalis	ATCC 49417	1	-	NT
Porphyromonas cansulci	NCTC 12858^T	1	-	NT
Porphyromonas cangingivalis	NCTC 12856^T	1	-	NT
Porphyromonas canoris	NCTC 12835^T	1	-	NT
Porphyromonas somerae	ATCC BAA-1230^T	1	-	NT
Porphyromonas uenonis	ATCC BAA-906^T	1	-	NT
Porphyromonas gulae	ATCC 51700^T	1	-	NT
Tannerella forsythensis	ATCC 43037	1	NT	-
Bilophila wadsworthia	ATCC 49260^T	1	NT	+
Bilophila wadsworthia	ATCC 51581	1	NT	+
Bilophila wadsworthia	Clinical isolates	8	NT	+
Desulfovibrio piger	ATCC 29098^T	1	NT	
Desulfovibrio piger	Clinical isolates	4	NT	-
Desulfovibrio desulfuricans	ATCC 7757	1	NT	-
Desulfovibrio desulfuricans	Clinical isolates	4	NT	-
Sutterella wadsworthensis	ATCC 51579^T	1	NT	-
Desulfovibrio fairfieldensis	Clinical isolates	2	NT	-
Desulfovibrio vulgaris	Clinical isolates	2	NT	-

NT: not tested

Gram-positive anaerobes:

The specificities of the primer and probe sets for *F. magna, M. micros* and *P. anaerobius* were tested by running PCR with DNA samples from 14 type strains and 17 well-characterized clinical isolates that are phylogenetically related to GPAC species; this resulted in amplification only with the DNA from the corresponding GPAC reference strain(s). None of the non-corresponding organisms tested by the PCR showed an increase in fluorescence to indicate cross-activity with the species-specific primer/probe sets (See table 4).

Comparison of the sequences of primer/probe set for *Actinomyces odontolyticus* to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility predicted it cross-reacts with *A. lingnae*. Further tests will be done with this set of oligonucleotides in the future.

Table 4: Bacterial strains tested by TaqMan assay

Species	Strains	No.	Fmag-F/R/P	Pmic-F/R/P	Pana-F/R/P
Finegoldia magna	CCUG 17636 ^{T}	1	+	-	-
Finegoldia magna	Clinical isolates	6	+	-	-
Micromonas micros	ATCC 33270 ^{T}	1	-	+	-
Micromonas micros	Clinical isolates	6	-	+	-
Peptostreptococcus anaerobius	CCUG 7835 ^{T}	1	-	-	+
Peptostreptococcus anaerobius	Clinical isolates	5	-	-	+
Anaerococcus hydrogenalis	ATCC 49630 ^{T}	1	-	-	-
Anaerococcus lactolyticus	CCUG 31351 ^{T}	1	-	-	-
Anaerococcus octavius	CCUG 38493 ^{T}	1	-	-	-
Anaerococcus prevotii	CCUG 41932 ^{T}	1	-	-	-
Anaerococcus tetradius	CCUG 46590 ^{T}	1	-	-	-
Anaerococcus vaginalis	CCUG 31349 ^{T}	1	-	-	-
Peptoniphilus. asaccharolyticus	CCUG 9988 ^{T}	1	-	-	-
Peptoniphilus harei	CCUG 38491 ^{T}	1	-	-	-
Peptoniphilus indolicus	CCUG 17639 ^{T}	1	-	-	-
Peptoniphilus ivorii	CCUG 38492 ^{T}	1	-	-	-
Peptoniphilus lacrimalis	CCUG 31350 ^{T}	1	-	-	-

Gram-positive aerobes:

Anginosus group and Group A and B streptococci:

As the non-anginosus group streptococci, the 14 reference strains representing 12 different species (**Table 5**) were used. As the anginosus streptococci, the type strain (CCUG 27298^T) and three isolates of *S. anginosus*, the type strain (ATCC 27283 ^{T}), one reference strain (CCUG 24889) and one isolate of *S. constellatus subsp. constellatus*, and the type strain (CCUG 32759 ^{T}) and two isolates of *S. intermedius* were used. The clinical strains of the anginosus group were recovered from our facility. The clinical isolates were identified by routine biochemical methods, which included evaluation by use of the Vitek and API systems. None of the 14 non-anginosus organisms tested by the PCR showed an increase in fluorescence to indicate cross-reactivity with the primer/probe sets (Table 5). Conversely, all the anginosus streptococci isolates showed an exponential increase in fluorescence. Similarly, the primer/probe sets for Group A and Group B streptococci were tested against a battery of streptococci (Table 5), and fluorescent signal were only detected from corresponding strains.

***Staphylococcus* spp. & *S. aureus*:**

An analysis of the bacterial battery demonstrated the specificities of our primers and probes. The broad-range primer/probe set designed for the *Staphylococcus* genus generated positive curves for all of the staphylococci tested (**Fig.2.**). The *S. aureus*-specific assay detected only the *S. aureus* isolates in the battery (**Table 5**).

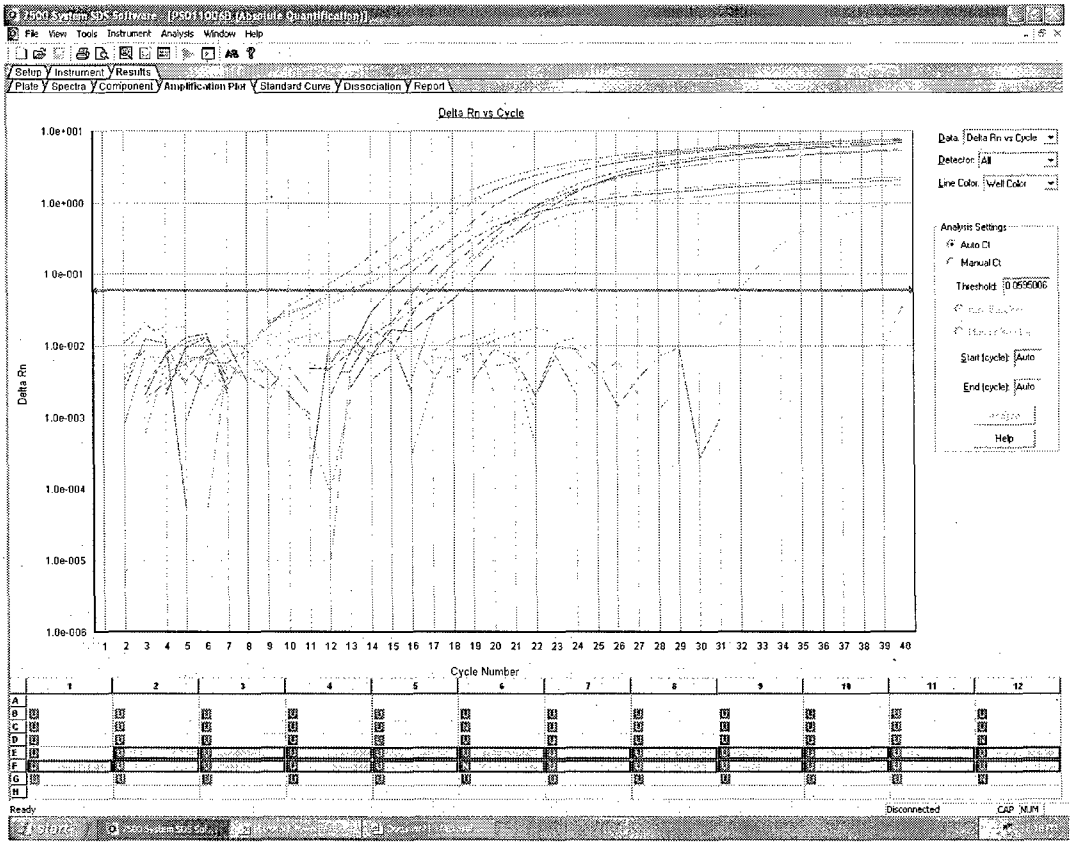

Fig. 2. Example showing the FAM fluorescence detection of *Staphylococcus* spp.

Gram-negative aerobe:

The specificity of the primer/probe set devised for *Pseudomonas aeruginosa* was predicted by comparison to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility and was compared to all available 16S rDNA sequences by using the BLAST database search program. Theoretical cross-specificity analysis indicated that the detection system would be specific for *Pseudomonas aeruginosa* at the species level. The primer/probe set was tested against two *Pseudomonas aeruginosa* strains (type strain ATCC 10145 and reference strain ATCC 27853) and the other two *Pseudomonas* strains including the type strain of *Pseudomonas fluorescens* (ATCC 13525^T) and the type strain of *Pseudomonas putida* (ATCC 12633 ^{T}). The fluorescent signal was only detected from *Pseudomonas aeruginosa* strains. Specificity of the primer/probe set needs to be further confirmed by testing more phylogenetically related strains in the future.

Table 5. Bacterial strains tested by TaqMan assay

Species	Strains	No.	Staphy-F/R/P	Saur-F/R/P	GAS-F/R/P	GBS-F/R/P	AGS-F/R/P
Staphylococcus spp.							
S. aureus	ATCC 29213	1	+	+	NT	NT	NT
S. aureus	ATCC 25923	1	+	+	NT	NT	NT
S. aureus	Clinical isolates	4	+	+	NT	NT	NT
S. epidermidis	ATCC 12228	1	+	-	NT	NT	NT
S. epidermidis	ATCC 49134	1	+	-	NT	NT	NT
S. epidermidis	Clinical isolates	2	+	-	NT	NT	NT
S. haemolyticus	ATCC 29970^T	1	+	-	NT	NT	NT
S. haemolyticus	WAL 1020	1	+	-	NT	NT	NT
S. hominis	WAL 937	1	+	-	NT	NT	NT
S. lugdunensis	ATCC 49576	1	+	-	NT	NT	NT
S. saprophyticus	ATCC 15305^T	1	+	-	NT	NT	NT
S. simulans	ATCC 27851	1	+	-	NT	NT	NT
S. warneri	ATCC 49454	1	+	-	NT	NT	NT
S. warneri	WALA 939	1	+	-	NT	NT	NT
S. xylosus	ATCC 29971^T	1	+	-	NT	NT	NT
CoNS *Staphylococcus*	Clinical isolates	7	+	-	NT	NT	NT
Streptococcus spp.							
S. bovis	ATCC 35034	1	-	NT	-	-	-
S. dysgalactiae subsp. *equisimilis*	ATCC 35666	1	NT	NT	-	-	-
S. mitis	ATCC 49456^T	1	-	NT	-	-	-
S. mutans	ATCC 25175^T	1	-	NT	-	-	-
S. oralis	ATCC 9811	1	NT	NT	-	-	-
S. salivarius	ATCC 7073^T	1	-	NT	-	-	-
S. sanguinis	ATCC 10556^T	1	-	NT	-	-	-
S. vestibularis	ATCC 49124^T	1	-	NT	-	-	-
S. anginosus	CCUG 27298^T	1	NT	NT	NT	-	+
S. anginosus	Clinical isolates	3	NT	NT	NT	NT	+
S. agalactiae	ATCC 12386	1	NT	NT	-	+	-
S. agalactiae	ATCC 13813^T	1	NT	NT	-	+	-

S. agalactiae	Clinical isolates	2	NT	NT	NT	+	NT
S. iniae	ATCC 29178^T	1	NT	NT	NT	-	-
S. constellatus subsp. *constellatus*	ATCC 27823^T	1	NT	NT	-	-	+
S. constellatus	CCUG 24889	1	NT	NT	NT	-	+
S. constellatus	WALA 154	1	NT	NT	NT	NT	+
S. intermedius	CCUG 32759	1	NT	NT	NT	NT	+
S. intermedius	Clinical isolates	2	NT	NT	NT	NT	+
S. pneumoniae	ATCC 35088	1	NT	NT	-	-	-
S. pneumoniae	ATCC 49615	1	NT	NT	NT	-	NT
S. pneumoniae	ATCC 6305	1	NT	NT	NT	-	NT
S. pyogenes	ATCC 12384	1	NT	NT	+	-	-
S. pyogenes	ATCC 19615	1	NT	NT	+	-	-
S. pyogenes	Clinical isolates	2	NT	NT	+	-	NT

NT: not tested

KEY RESEARCH ACCOMPLISHMENTS

Rapid identification of pathogens involved in wound infections is critical for successfully controlling, managing and eradicating this type of infection. Rapid field-based tests to detect the pathogens involved in the infections are not available. The most significant accomplishment during the last year was development of a rapid real-time TaqMan PCR assay for most of the pathogens encountered in wound infections. This rapid and specific assay provides a new diagnostic tool that conceivably will redefine wound infection management and control strategies for this important problem in both military and civilian life. Evaluation of the sensitivity of the assay is underway.

REPORTABLE OUTCOMES

When the entire set of primers and probes has been completed, this information plus the methodology for real-time PCR using these will be published.

CONCLUSION

We have developed a real-time PCR based high throughput detection method for certain important wound pathogens.

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