Real-time PCR assays compared to culture-based approaches for identification of aerobic bacteria in chronic wounds

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Abstract

Chronic wounds cause substantial morbidity and disability. Infection in chronic wounds is clinically defined by routine culture methods that can take several days to obtain a final result, and may not fully describe the community of organisms or biome within these wounds. Molecular diagnostic approaches offer promise for a more rapid and complete assessment. We report the development of a suite of real-time PCR assays for rapid identification of bacteria directly from tissue samples. The panel of assays targets 14 common, clinically relevant, aerobic pathogens and demonstrates a high degree of sensitivity and specificity using a panel of organisms commonly associated with chronic wound infection. Thirty-nine tissue samples from 29 chronic wounds were evaluated and the results compared with those obtained by culture. As revealed by culture and PCR, the most common organisms were methicillin-resistant *Staphylococcus aureus* (MRSA) followed by *Streptococcus agalactiae* (Group B streptococcus) and *Pseudomonas aeruginosa*. The sensitivities of the PCR assays were 100% and 90% when quantitative and qualitative culture results were used as the reference standard, respectively. The assays allowed the identification of bacterial DNA from ten additional organisms that were not revealed by quantitative or qualitative cultures. Under optimal conditions, the turnaround time for PCR results is as short as 4–6 h. Real-time PCR is a rapid and inexpensive approach that can be easily introduced into clinical practice for detection of organisms directly from tissue samples. Characterization of the anaerobic microflora by real-time PCR of chronic wounds is warranted.

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Introduction

Chronic wounds cause substantial morbidity, engender costs over $10 billion annually in the USA [1] and especially impact diabetics [2,3]. Chronic wounds have been ascribed to a variety of causes, most prominently poor vascular supply and infection. Routine cultures that are used to diagnose bacterial colonization and infection are time-consuming, and may not fully reveal the microbiological communities within these wounds because cultures often lack sensitivity in polymicrobial environments [4]. As such, molecular diagnostic tools that allow reliable and consistent identification of all pathogens, especially anaerobes, are highly desirable.

As an adjunct to cultures, PCR-based methods have been evaluated for the detection of bacteria in blood [5–7], joint fluid [8] cerebrospinal fluid [9], heart tissue [10] and burn wounds [11]. Previous molecular analyses of chronic wound microflora were based on the initial amplification of bacteria with universal primers for 16S rRNA genes followed by specific identification approaches. These approaches included sequencing of PCR products [12], denaturing gradient gel electrophoresis followed by cloning and sequencing [13] and pyrosequencing [14–16]. These identification techniques, however, are costly, require sophisticated instrumentation, and therefore are not easily translatable into clinical practice.

Real-time PCR is faster than traditional PCR and does not require post-amplification manipulation for bacterial identification [6–8]. Application of this molecular approach to date has been limited to the detection of specific bacterial species from

tissue [11] or infections, such as bacterial meningitis, typically caused by a limited number of known pathogens [9] because there are technical challenges associated with multiplex real-time PCR. To overcome these limitations, 16S rRNA gene probes targeting multiple species have been designed, and melt curve analyses have been performed to identify the species based on characteristic melt curve profiles [6,17].

As an initial step toward the development of a molecular diagnostic tool for the detection of aerobic and anaerobic organisms, we have developed a series of real-time PCR assays targeting 14 of the most common and clinically relevant aerobic bacteria in chronic wounds. The sensitivity and specificity of the assays was evaluated using a well-characterized set of chronic wound samples [16,18]. Although our assays targeted a limited number of aerobic organisms, this approach could be expanded to include other clinically relevant organisms, including anaerobes.

Materials and Methods

Bacterial species and spiked samples

The analytical sensitivity and specificity of the PCR assays was tested using a panel of 39 reference and clinical strains (Table S1). The panel of bacterial species was selected because they were either organisms targeted by the PCR assays or organisms frequently isolated from chronic wounds at the Johns Hopkins Wound Center (Baltimore, MD, USA) [18]. Tissue known to be free of any of the targeted organisms was used as a negative control.

To determine the limit of detection (LOD) of each assay, serial dilutions ranging from 10^7–10^1 CFU/mL of each of the target organisms were prepared. Each of the serial dilutions was spiked with 20 mg of the previously described bacterium-free tissue and extracted for total DNA as described below. The LOD was calculated based on CFU/mL.

Clinical specimens

Tissue samples from chronic wounds were collected, using a 3-mm curette, from 28 patients attending the Johns Hopkins Wound Center, as previously described [16,18]. To allow for multiple tests to be performed, tissue samples were divided into three portions: one was evaluated by qualitative culture in Clinical Laboratory Improvement Amendments-certified laboratories, the second portion was analysed by quantitative culture and real-time PCR and the third was used for metagenomic analysis [16]. Qualitative and quantitative cultures were carried out in accordance with established protocols [18]. The study was approved by the Johns Hopkins Institutional Review Board.

DNA extraction

Each sample was processed separately in a DNA-free biological safety cabinet. Tissue homogenate (200 μL) was extracted for total DNA with the DNeasy Blood and Tissue DNA purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Primer and probe design

Three new 16S rRNA gene PCR assays were designed to target *Acinetobacter baumannii, Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis/vulgaris* and *Pseudomonas aeruginosa* (Table 1). The *Enterococcus/Streptococcus* assay and *Staphylococcus aureus* assays have been described previously [6,19]. A previously validated methicillin-resistant *S. aureus* (MRSA) PCR assay that can differentiate between MRSA and methicillin-resistant coagulase-negative staphylococci (CoNS) was used with minor modifications [20]. For detection of Group B streptococci (GBS), the previously described forward primer TTTCACCAGCTGT ATTAGAAGTA [21] was used in combination with the newly designed reverse primer GTTCCCTGAACATTATCTTT GAT. All primers and probes were synthesized by TIB Molbiol (Adelphia, NJ, USA).

Real-time PCR assays

All assays were carried out in two runs: one run included all probe-based assays (Table 1) and the other included the SYBR Green-based assays (GBS and MRSA). PCR reactions were carried out individually, but in parallel, in 96-well plates in 20-μL reactions consisting of 10 μL of 2× LightCycler 480 Probes Master/SYBR Green Master, 1 μM of each primer and probe (if necessary), 5 μL of extracted DNA and PCR-grade water to a final volulme of 20 μL. Every run included positive and negative controls. The amplification parameters included steps at 95°C for 10 min, followed by 35 cycles at 95°C for 10 s, 55°C for 10 s and 72°C for 10 s, followed by probe melting, which consisted of a continuous fluorescence reading from 50–85°C at five acquisitions per 1°C/s. PCR reactions were carried out in duplicates on the LightCycler 480 System (Roche Applied Science, Indianapolis, IN, USA).

Post-PCR analysis

Samples were considered to be positive for the targeted species if they yielded an amplification curve three cycles below the negative control, and had a characteristic melt curve profile similar to that of the positive control. Given the controversy regarding the adequacy of qualitative culture assessment [18], we evaluated the overall sensitivity and specificity of the PCR assays compared to qualitative and

TABLE 1. Primers and probes used in the present study

Specificity	Forward primer	FL probe	LC probe	Reverse primer
A. baumannii *P. aeruginosa*	ATGAGCCTARGTCGGATTAGCT	CACTTTAAGCGAGGAGGAGGCTACT-FL ACTTTAAGTTGGGAGGAAGGGC-FL	LC-Red-610-CTAGAGATAGTGGACGTTACTCGC-Ph LC670GTAAGTTAATACCTTGCTGTTTTGACGTTACCAPh	AGTTAGCCGGTGCTTATTCTG
E. coli	AACTGGAGGAAGGTGGGGAT	CCTCATAAAGTGCGTCGTAGTCCG -FL	LC-640-ATTGGAGTCTGCAACTCGACTCCA -Ph	AGGAGGTGATCCAACCGCA
K. oxytoca	C	AT		
K. pneumoniae		AT		
M. morganii	C	A A		
P. mirabilis	C	A AT		
P. vulgaris	C	A AT		
K. oxytoca	CAGCAGCCGCGGTAATAC	GGCTAGAGTCTTGTAGAGGGGGGTAGA -FL G	LC-610-TTCCAGGTGTAGCGGTGAAATGC -Ph	CGTGGACTACCAGGGTATCTAAT
K. pneumoniae				
S. marcescens		A C		
S. aureus[a]	GATTGATGGTGATACGGT	GTTTGACAAAGGTCAAAGAACTGATAAAT-FL	LC-610-TGGACGTGGCTTAGCGTATATTTAT-Ph	CAAGCCTTGACGAACTA
E. faecalis[b] *E. faecium* *S. pneumoniae* *S. pyogenes*	CGTGAGATGTTGGGTTAAGTC	TCTAGCGACTCGTTGTACTTCCCATTGT-FL	LC-670-GCACGTGTGTAGCCCAGGTCATAAG-Ph	GCTGATCCGCGATTACTAGC

FL, Fluorescein; LC-Red560, LightCycler 560-N-hydroxy-succinimide ester; [Ph], 3'-phosphate. All primers and probes are labeled in the 5' - 3' direction.
[a]Assay adapted from Costa et al, 2005 [19].
[b]Assay adapted from Wellinghausen et al, 2004 [6].
16S DNA sequences were a perfect match to primers and probes unless noted by sequence variance unique to the specific species.

quantitative culture results as reference standards. The total possible number of PCR-identifiable organisms (n = 546) was used to calculate the specificity of the PCR assays and was based on the notion that the PCR assays can detect up to 14 different species in each of the 39 samples. A 95% CI based on binomial distribution was used to provide inferences for clinical sensitivity and specificity.

Results

Analytical sensitivity and specificity of the PCR assays using spiked samples

Each of the assays correctly and exclusively identified their respective target organisms. The LOD of each assay was in the range 10^1–10^2 CFU/mL. Species were differentiated by their unique melt curve profile (Fig. 1a) resulting from DNA sequence variations in the binding region of the FL probe (Table 1). Although *Klebsiella* and *Proteus* can be easily differentiated, specific species of these genera with identical DNA sequences (Table 1) could not be differentiated by their melt curve profile (Fig. 1a). A secondary PCR assay, which can simultaneously detect *Serratia marcescens* was designed to differentiate *K. pneumoniae* from *K. oxytoca* (Fig. 2). A PCR assay to differentiate *P. mirabilis* from *P. vulgaris* was not available for this study. To evaluate the theoretical sensitivity and specificity of using a single probe for the identification of multiple species, we analysed a spiked sample containing *E. coli, K. oxytoca, M. morganii* and *P. mirabilis* with the PCR assay targeting these four species. As shown in Fig. 1b, this PCR assay can simultaneously identify up to three related species with a single set of hybridization probes.

Clinical samples and culture-based results

Thirty-nine samples were collected, in 39 wound visits, from 28 subjects (16 males and 12 females, age range 33–83 years); 15 (54%) patients were diabetic. Primary ulcer diagnoses were neuropathic (39%), decubitus and/or pressure (21%), venous (15%), traumatic and/or surgical (11%) and other (14%). Ninety-seven percent (38/39) of wound samples were positive for at least one organism by culture, and 60% of samples had three or more organisms. The most prevalent organisms identified by culture were MRSA (42%) followed by *P. aeruginosa* (28%) and Group B streptococcus (21%). Methicillin-sensitive *S. aureus* was present in 17% of the wounds. Anaerobes (*Prevotella, Bacteroides* and *Peptostreptococcus*) accounted for 11% (10/92) of the organisms cultured and were recovered from 21% (8/39) of the wound samples (Table 2). Of the 73 organisms isolated by quantitative cultures (Table 2), quantitative data (CFU/g) were available for 70 of the organisms, and 90% (63/70) of these organisms were present at $\geq 10^5$ CFU/g of tissue.

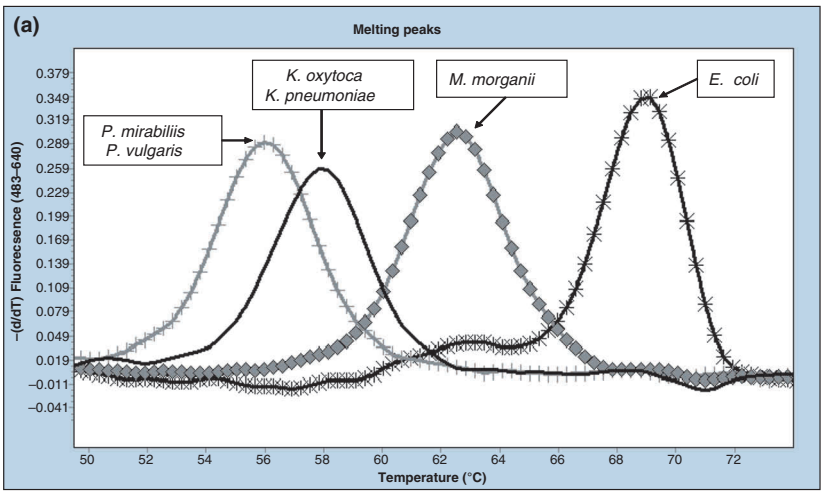

FIG. 1. (a) Differentiation of four different species by melt curve analysis. For simplicity, the negative control has been omitted. (b) Identification of three different species by a single probe from a single sample.

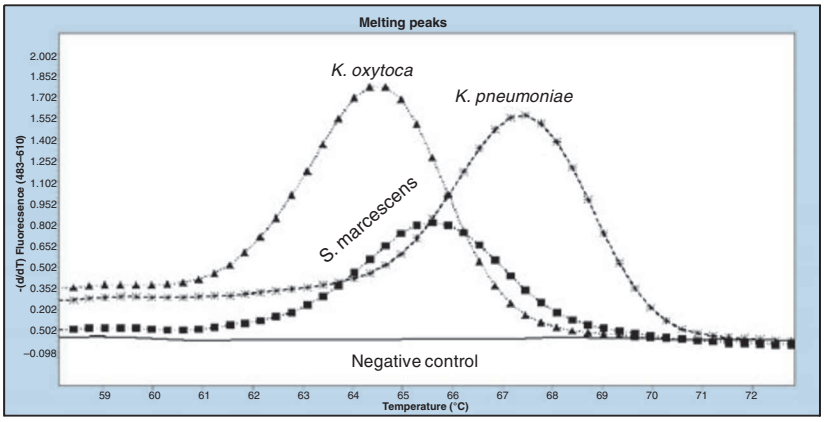

FIG. 2. Differentiation of *Klebsiella oxytoca* from *Klebsiella pneumoniae*.

Diagnostic sensitivity and specificity of the PCR assays with clinical samples

Eighty-two percent (31/38) of the culture-positive wound samples had organisms that were identifiable by the PCR assays. The remaining seven samples were colonized by CoNS and/or *Corynebacterium* (6), and Group G streptococci (1). Overall, the PCR assays targeted 73% (60/82) and 75% (55/73) of the aerobic organisms isolated by qualitative and quantitative cultures, respectively. The majority of the aerobic organisms not targeted by the PCR assays were CoNS

TABLE 2. Numbers of isolates/microorganisms detected by qualitative and quantitative culture and by PCR

Culture result and PCR availability	Qual. only	Quant. only	Any culture	Any Culture and PCR	Both culture	Both Culture and PCR	PCR only
PCR available							
MRSA	15	16	16	16	15	15	2
Pseudomonas aeruginosa	10	10	10	10	10	10	0
Group B streptococcus	8	7	10	10	5	5	1
Staphylococcus aureus	6	6	6	6	6	6	0
Proteus mirabilis	5	3	5	3	3	3	0
Acinetobacter baumannii	3	2	3	3	2	2	0
Enterococcus faecalis	3	2	3	3	2	2	0
Escherichia coli	3	4	5	4	2	2	1
Klebsiella pneumoniae	3	1	3	1	1	1	1
Morganella morganii	2	2	3	3	1	1	2
Enterococcus faecium	1	1	1	1	1	1	0
Streptococcus spp.	1	0	0	0	0	0	0
Klebsiella oxytoca	0	1	1	1	0	0	3
Serratia marcescens	0	0	0	0	0	0	0
Subtotal	**60**	**55**	**66**	**61**	**48**	**48**	**10**
PCR not available							
Aerobic organisms							
Corynebacterium	7	6	12		6		
CoNS	6	5	6		5		
Enterobacter cloacae	2	2	3		1		
Providencia stuartii	1	3	3		1		
Group G streptococcus	2	2	2		2		
Viridans Group Streptococcus	2	0	2		0		
Alcaligenes Species	1	0	1		0		
Citrobacter freundii	1	0	1		0		
Subtotal	**22**	**18**	**30**		**15**		
Total aerobic organisms	**82**	**73**	**96**		**63**		
Anaerobic organisms							
Prevotella Species	4	0	4		0		
Bacteroides Species	3	0	3		0		
Peptostreptococcus Species	3	0	3		0		
Total anaerobic organisms	**10**	**0**	**10**		**0**		
Total	**92**	**73**	**106**		**63**		

MRSA, methicillin-resistant *S. aureus*; Qual. = Qualitative culture; Quant. = Quantitative culture; CoNS = Coagulase-negative staphylococci.

and *Corynebacterium* (Table 2). The PCR assays correctly identified 54 of the 60 aerobic organisms isolated by qualitative cultures, and 55 of the 55 organisms recovered by quantitative cultures (Table 3A). The PCR assays correctly identified 100% (48/48) and 92% (61/66) of the aerobic organisms isolated by the two culture methods, and by either of the two culture methods, respectively (Table 2). The calculated overall sensitivity and specificity of the PCR assays in comparison to the quantitative culture results as the reference standard were 100% (55/55) (95% CI 93.5–100%) and 96.7% (475/491) (95% CI 94.8–98.1%), respectively. The PCR assays were 90.0% (54/60) sensitive (95% CI 79.5–96.2%) and 96.5% (469/486) (95% CI 94.5–98.0%) specific in comparison to qualitative culture results.

Discordant species-specific PCR and culture results

Six organisms from six samples yielded discordant qualitative culture and PCR results (Table 3B2). *E. coli* (WS34), Group A streptococcus (WS40), *K. pneumoniae* (WS12) and *P. mirabilis* (WS27) were all reported as showing minimal growth during qualitative analysis, but were negative by quantitative cultures and PCR.

Two isolates [*K. pneumoniae* (WS17) and *P. mirabilis* (WS38)] were reported as showing heavy growth by qualitative culture but were PCR- and quantitative culture-negative.

The PCR assays identified DNA from ten organisms that were not reported by qualitative or quantitative cultures (Table 3B1). Additionally, there were seven samples with qualitative culture-negative, quantitative culture-positive and PCR-positive results (Table 3B2), and six samples with qualitative culture-positive, quantitative culture-negative and PCR-positive results (Table 3B3).

Antimicrobial therapy and microbial wound content

To determine whether antimicrobial therapy impacted assay performance, we compared the culture and PCR results for subjects recently treated with antibiotics and those untreated. An average of 3 and 2.1 isolates were recovered by culture from untreated and treated wounds, respectively. There was no difference in the number of isolates (2.2) recovered by culture from treated wounds regardless of whether the last dose of antibiotic was taken 24 h or 2 weeks prior to sample collection. However, 46% (6/13) of the wounds that were treated within 24 h of sample collection were either colonized with CoNS or *Corynebacterium* (5) or were culture-negative (1). Eighty percent (8/10) of the organisms exclusively detected by PCR were from untreated wounds (Table 3B1).

TABLE 3. Concordance and discordance of qualitative and quantitative culture results in comparison to species-specific PCR results

A. Comparison of culture and PCR results stratified by culture

Qualitative culture					Quantitative culture				
	Culture positive		Culture negative/PCR positive			Culture positive		Culture negative/PCR positive	
# of isolates[a]	PCR		17		# of isolates[a]	PCR		16	
	+	-	No abx	Abx use		+	-	No abx	Abx use
60	54	6	15	2	55	55	0	13	3

B. Discordant culture and species-specific PCR

ID #	Number of samples	Organism	Qualitative culture	Quantitative culture[b]	PCR	Antibiotic use within 2 weeks	Comments
1. Discordant culture and species-specific PCR							
WS38[c]	1	*Escherichia coli*	-	-	+	Yes	Topical treatment within 24 hours of sample collection
WS17[c]	1	Group B streptococcus	-	-	+	No	
WS35[c], WS40	2	*Klebsiella oxytoca*	-	-	+	No	
WS11[c]	1	*Klebsiella pneumoniae*	-	-	+	No	Sample stored at -70°C for 3 days prior to analysis by quantitative culture.
WS 39	1	*Klebsiella oxytoca*	-	-	+	Yes	Bactrim DS within 24 hour of sample collection
WS37[c, d], WS41[c, d]	2	*Morganella morganii*	-	-	+	No	
WS4[c], WS12[c]	2	MRSA	-	-	+	No	
2. Discordant qualitative culture and species-specific PCR							
WS34	1	*Escherichia coli*	+	-	-	No	Minimal growth reported
WS40	1	Group A streptococcus	+	-	-	No	Minimal growth reported
WS17[c]	1	*Klebsiella pneumoniae*	+	-	-	No	Heavy growth reported
WS27[c], WS38[c]	2	*Proteus mirabilis*	+	-	-	No	Minimal and heavy growth reported for wounds # 27 and 38, respectively.
WS12[e]	1	*Klebsiella pneumoniae*	+	-	-	No	Minimal growth reported
	1	*Klebsiella oxytoca*	-	2.0×10^7	+	No	
WS11[c], WS 29	2	*Escherichia. coli*	-	5.7×10^5	+	No	
WS37[c, d], WS 41[c, d]	2	Group B streptococcus	-	3.3×10^7	+	No	
WS 34	1	*Morganella morganii*	-	4.5×10^5	+	No	
WS13	1	MRSA	-	1.3×10^7	+	No	
3. Discordant quantitative culture and species-specific PCR							
WS13[c]	1	*Acinetobacter baumannii*	Few	-	+	No	Six species isolated from this wound.
	1	*Morganella morganii*	Few	-	+	No	
WS11[c]	1	*Enterococcus faecalis*	Few	-	+	No	Isolated from thioglycollate medium
WS8, 34[c]	2	Group B streptococcus	Few	-	+	No	
WS32	1	Group B streptococcus	Few	-	+	Yes	Bactrim DS within 2 weeks of sample collection

[a]Excludes isolates for which PCR assays were not available. Please refer to Table 2 for a list of organisms.
[b]CFU/gm of tissue.
[c]Three or more organisms cultured from this wound.
[d]Two wounds from one patient simultaneously sampled at three sites (#37) and two sites (#41).
[e]Reported as *Klebsiella pneumonia* by qualitative culture; identified as *Klebsiella oxytoca* by quantitative culture and PCR. Isolate recovered by qualitative culture was not available for re-testing.
Abx, antibiotics; MRSA, methicillin-resistant *Staphylococcus aureus*

Discussion

Real-time PCR assays that can simultaneously target multiple species may be a useful adjunct for the rapid and accurate identification of bacterial pathogens in chronic wounds. The only commercially available, multi-species PCR-based test, Septi*Fast* [22–24], has not been applied to chronic wounds and is not available in the USA. Using our own panel of PCR assays and chronic wound samples, we demonstrated the utility of using a targeted multi-species, real-time PCR approach for the rapid detection of bacterial species directly from tissue.

As a proof of principle, we designed a panel of PCR assays to target the most clinically relevant aerobic bacterial species commonly isolated from chronic wounds based on culture results from our patient population and previous studies. *S. marcescens*, which is not frequently isolated from chronic wounds, was not purposely targeted, but could be detected by one of the assays. Although CoNS and *Corynebacterium* are frequently isolated from chronic wounds, PCR assays targeting these organisms were not included in the panel because they are not believed to be clinically significant in the setting of chronic wounds. PCR assays targeting *Alcaligenes* spp., *Citrobacter freundii*, *Enterobacter cloacae*, Group G streptococci, *Providencia stuartii* and Viridans group streptococci were not included in the panel of assays because, as demonstrated in the current study, they are not commonly isolated from the chronic wounds of our patients (Table 2). However, some of these organisms are clinically relevant and should be targeted in future studies.

The sensitivity of our PCR assays was excellent compared to that of cultures. The assay system is versatile because it can be run individually or as a panel; additional assays targeting other clinically relevant bacterial species, including anaerobes can easily be added to a panel, and can be adapted to other biological materials such as blood. Under optimal conditions, the turnaround time for PCR results can be as short as 4–6 h.

A total of six qualitative culture-positive, PCR-negative samples were noted. There are two possible explanations for these results. (i) Positive cultures resulting from laboratory contamination. None of the isolates were revealed by

quantitative cultures and most of them showed minimal growth during qualitative analysis. (ii) A lack of sufficient DNA for amplification or excess background DNA. It has been suggested that PCR fails to reach its theoretical sensitivity because of the use of a small sample volume after a series of enzymatic inductions of cell lysis, which may or may not effectively isolate the microbial DNA [25].

The PCR assays identified DNA from ten organisms that were not revealed by either qualitative or quantitative cultures. Recent antibiotic treatment of the patients is unlikely to be the reason for the discordant results because the majority of them had not received topical or systemic therapy within the 2 weeks prior to sample collection. There are several possible explanations for the culture-negative, PCR-positive results, including: (i) a lack of sensitivity of cultures in polymicrobial settings, which has been previously reported in the setting of chronic wounds [4]; (ii) molecular detection of nonviable bacteria; (iii) false-positives resulting from cross-reactivity with other species that were not cultured and that were not part of the panel used to determine the specificity of the PCR assays.

To further expand the applicability of this targeted approach, we have conducted preliminary studies with previously reported real-time PCR assays targeting the *Bacteroides* spp. and *Prevotella* spp. [26,27] but have found them to lack specificity in the setting of chronic wounds. Given that anaerobes such as *Bacteroides* and *Prevotella* comprised a large portion of the chronic wound microflora communities [14–16] and that these species cannot be easily isolated by culture, real-time PCR assays targeting these anaerobes as well as *Peptostreptococcus* spp. should be designed and routinely used in the characterization of the microflora of chronic wounds. We are currently working on developing assays targeting several anaerobic species.

In conclusion, we demonstrated that a targeted real-time PCR approach can be used for the rapid detection of the most prevalent cultivable, aerobic organisms isolated from chronic wounds. Additionally, this approach is fast and uses instrumentation that is becoming more readily available in the clinical setting. Subsequent to the development of additional assays targeting other clinically relevant aerobic and anaerobic organisms, we look forward to developing a rapid, cost-effective, clinically applicable molecular diagnostic panel to serve in the diagnosis and care of chronic wounds.

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Transparency Declaration

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Control strains.

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