

Molecular Detection of *Streptococcus pyogenes* and *Streptococcus dysgalactiae* subsp. *equisimilis*

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Abstract We developed molecular diagnostic assays for the detection of *Streptococcus pyogenes* (GAS) and *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE), two streptococcal pathogens known to cause both pharyngitis and more invasive forms of disease in humans. Two real-time PCR assays coupled with an internal control were designed to be performed in parallel. One assay utilizes a gene target specific to GAS, and the other utilizes a gene target common to the two species. Both assays showed 2–3 orders of magnitude improved analytical sensitivity when compared to a commercially available rapid antigen test. In addition, when compared to standard culture in an analysis of 96 throat swabs, the real-time PCR assays resulted in clinical sensitivity and specificity of 91.7 and 100%, respectively. As capital equipment costs for real-time PCR can be prohibitive in smaller laboratories, the real-time PCR assays were converted to a low-density microarray format designed to function with an inexpensive photopolymerization-based non-enzymatic signal amplification (NESATM) method. *S. pyogenes* was successfully detected on the low-density microarray in less than 4 h from sample extraction through detection.

Keywords *Streptococcus pyogenes* ·
Streptococcus dysgalactiae subspecies *equisimilis* ·

Real-time PCR · Low-density microarray ·
Molecular diagnostics · Pathogen detection

Introduction

Group A streptococcal (GAS) infections caused by *Streptococcus pyogenes* are responsible for a wider variety of human infections than any other bacterium [1]. A strictly human pathogen, GAS causes noninvasive infection usually in the form of pharyngitis (‘strep’ throat) and impetigo [1, 2]. Pharyngitis accounts for approximately 1–2% of all visits to primary physicians in the United States each year [3, 4], with a total of ~40 million cases reported annually [5]. Although not as common as GAS, *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) of Lancefield serological groups C and G [6] has also been shown to be a causative agent of pharyngitis in both children and adults [5, 7–15]. SDSE found in most human infections expresses either the group G or group C antigen (and more infrequently the A and L antigens) [16], and historically was referred to as “group C Strep” or “group G Strep”. The importance of SDSE in streptococcal pharyngitis was highlighted by a recent report in which 11% of 306 patients presenting with pharyngitis were infected with SDSE [12]. These authors recommend that SDSE be considered a throat pathogen in line with GAS. It has also been noted that, although most research to date has focused on GAS, streptococci of groups C and G are being increasingly recognized as important human pathogens in a variety of other skin and more invasive infections including cellulitis, necrotizing fasciitis, toxic shock syndrome, and several others [17–19]. In fact, a recent study aimed at surveillance of invasive strep infections in Denmark showed that of 1,237 cases of invasive infection examined, 40% were

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attributed to GAS and 38% were attributed to groups C and G strep [20].

The gold standard method for the laboratory identification of GAS for nearly 50 years has been culture on 5% sheep's blood agar [10]. GAS bacterial colonies are identified after ~24–48 h incubation, and the assay is sensitive and inexpensive [3, 10]. The main drawbacks to culture are the time required between specimen collection and result and the presumptive nature of the identification. A similar presumptive identification scheme is utilized for SDSE. Rapid immunoassays (RIAs) for GAS have also become widely available. RIAs can provide an almost immediate result at the point of care, but have decreased clinical sensitivity compared to culture [10, 21, 22]. As a result, negative RIA results are often followed up by confirmatory culture to rule out the possibility of a false negative [23]. Currently available GAS RIAs do not detect infections due to SDSE. Considering the reduced sensitivity of RIAs compared to culture, instances of GAS infection with low bacterial load and all cases of SDSE would go undetected if only RIAs were used. Many recent reviews detail the merits of using molecular-based methods such as real-time PCR [24, 25] and low-density diagnostic microarrays [26–29] for the diagnosis of human pathogens. Two molecular-based tests for identification of GAS are commercially available [5, 22], but none are available for the direct identification of SDSE. Molecular-based detection of both GAS and SDSE would be beneficial for clinical diagnosis to enable timely administration of antibiotic therapy, and also for research purposes to further characterize the importance and prevalence of SDSE in human disease.

Herein we present the development and testing of both real-time PCR and low-density microarray assays for the detection of both GAS and SDSE. Two real-time PCR assays were developed. One assay detects only the presence of GAS, whereas the other assay is universal for both GAS and SDSE but does not amplify other potentially interfering streptococcal species. To our knowledge, this is the first hydrolysis probe-based real-time method that specifically detects the presence of SDSE. We determined the analytical sensitivity of the real-time PCR assays, and demonstrated a 2–3 order of magnitude improvement compared to a commercially available rapid immunoassay (RIA) for GAS. In addition, real-time PCR results from 96 clinical specimens were presented and compared to standard culture, resulting in clinical sensitivity and specificity of 91.7 and 100%, respectively. Lastly, proof-of-principle was demonstrated for the conversion of these real-time PCR assays to a low-density microarray format coupled with a new inexpensive non-enzymatic signal amplification method for detection. Although the focus of the present study was on the analysis of throat specimens, these same

assays should be applicable for the detection of GAS and SDSE in a wide range of clinical specimen types.

Methods and Materials

Sequence Design

A gene target unique to *S. pyogenes* that is chromosomally encoded and highly conserved across different strains was chosen as the GAS target. A common housekeeping gene was utilized to detect both GAS and SDSE but not differentiate them (hereafter referred to as the “GAS/SDSE co-detection gene target”). Enough sequence similarity in the housekeeping gene is present to enable co-amplification and detection of GAS and SDSE while discriminating against other closely related bacteria. The gene that codes for the 16s rRNA of the non-human pathogen *Bacillus subtilis* was chosen as an exogenous internal control. For all gene targets, available sequences (full and partial) were downloaded from the NCBI nucleotide database. BioEdit version 7.0.5.3 [30] was used for sequence visualization and manipulation. BLAST searches were conducted (<http://www.ncbi.nlm.nih.gov/BLAST/>) to evaluate potentially interfering organisms. Primers were designed using Primer3 software [31] with lengths between 18 and 25 nt (20 nt ideal), T_m between 57 and 63°C (60°C ideal), and GC content of 30–70%. The GAS only gene target primers produced an 80 nt amplicon, whereas the GAS/SDSE co-detection gene target produced a 99 nt product. These amplicons were kept short to enable efficient real-time PCR amplification, whereas the *B. subtilis* internal control amplicon (208 nt) was designed to be slightly longer to reduce competition for PCR reagents during amplification [32]. The amplicons generated with all three primer sets were initially analyzed on a 4% agarose gel (E-gel, Invitrogen Corp., Carlsbad, CA) alongside a Track-it™ 25-bp ladder (Invitrogen Corp., Carlsbad, CA), and the expected lengths were verified. The hydrolysis probes for real-time PCR were also designed with Primer3. Lengths were between 20 and 27 nt (22 nt ideal), T_m between 67 and 72°C (69°C ideal), and default settings were used for all other limits. Primer3 analyzes for possible self-dimerization and hairpin formation of primer pairs and probes and eliminates sequences not meeting strict requirements. Capture sequences for use on the low-density microarray were adapted from the hydrolysis probe sequences, using a 5'-amino-C6 modification for covalent attachment to aldehyde-functionalized glass slides. Sequence information for primers and hydrolysis probes is available upon request to not-for-profit enterprises for research use only.

Standards and Clinical Specimens

B. subtilis (internal control) and *S. pyogenes* (GAS) were both obtained from American Type Culture Collection (ATCC, Manassas, VA) as genomic DNA (#23857-D and #700294D-5, respectively). Three SDSE specimens were also obtained from ATCC as lyophilized culture material. Two of these SDSE specimens were Lancefield group C (#12388, #35666), and the other was Lancefield group G (#12394). The lyophilized culture material was rehydrated in 400 μ l of DNase, RNase-free water, and nucleic acid was then extracted from a 50 μ l aliquot using the Bacterial XpressTM nucleic acid extraction kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Eight cultured clinical specimens (suspected to be positive for GAS based on visual examination of culture results) were purchased from the Division of Laboratory Services at the Colorado Department of Public Health and Environment (Denver, CO). Several colonies were picked from each plate with a disposable loop and resuspended in 300 μ l of DNase, RNase-free water, and the nucleic acid was extracted as described earlier. The eight cultured specimens were also grouped using the Strep Plus kit (Oxoid Ltd., Hampshire, UK) through extraction of the Lancefield group-specific antigen (A, B, C, F, and G) using nitrous acid, and identification by latex agglutination. Seven of the clinical specimens were found to be positive for the Lancefield group A antigen, and one clinical specimen was Lancefield group G positive.

In addition, 96 throat swabs were generously donated by the Boulder Community Hospital (BCH) Microbiology Laboratory (Boulder, CO). For 18 of these throat swabs, a matched culture plate was also provided to enable further latex agglutination typing as described above. BCH first used the throat swabs to initiate a culture, and swabs were subsequently stored in a tube containing a sponge soaked in Liquid Stuart Medium, and kept refrigerated at 4°C until extracted. Culture was performed at BCH on Strep-selective agar using a four quadrant technique. Plates showing β -hemolytic growth were tested for the Lancefield group A antigen using latex agglutination, and specimens were grouped as "A" or "not-A" based on this result. In addition to the 'A'/not-A' designation, most cultures were also designated as '1+' (or 'rare'), '2+', '3+', or '4+', depending on which quadrant(s) showed bacterial growth. No further biochemical characterization was undertaken. The swabs were received by InDevR with no identifying information and in a blinded fashion. DNA was extracted from the swabs as described earlier, and a maximum of 11 specimens were extracted at one time along with an 'extraction negative' as a check for specimen cross-contamination during the extraction process.

Real-Time PCR

All primers for real-time PCR were ordered unmodified from Sigma-Genosys (St. Louis, MO), and all Taqman[®] hydrolysis probes targeting GAS and SDSE were ordered with 5'-FAM and 3'-BHQ1 modifications from Biosearch Technologies (Novato, CA). The internal control probe was ordered with 5'-CalFluor Orange 560 and 3'-BHQ1 modifications from Biosearch Technologies. Experiments were performed using the ROX, FAM, and HEX channels of the Mx3005p instrument (Stratagene, LaJolla, CA). All experiments used the FastStart Universal Probe Master Mix at a final 1 \times concentration (including ROX reference dye) (Roche Diagnostics, Indianapolis, IN). In addition, 2 μ l appropriate template was added to each reaction, using a final reaction volume of 25 μ l for all experiments.

The *B. subtilis* internal control primers were multiplexed with each gene target (two duplex amplifications) to include a check for PCR inhibition or failure. *B. subtilis* and the target organism were verified to coamplify even in cases where the *B. subtilis* was the more abundant species present, requiring that neither amplification reaction out-compete the other for reagents during the PCR reaction. In order to determine the limiting primer concentrations to reproducibly allow amplification of *B. subtilis* without sacrificing target amplification, a matrix of real-time PCR experiments at different primer concentrations was conducted for both gene targets [32]. The optimized GAS gene target assay utilized 400 nM GAS primers and 100 nM GAS probe. The optimized GAS/SDSE co-detection gene target assay used 1 μ M primers and 100 nM probe. Both reactions contained 200 nM *B. subtilis* internal control primers, 100 nM *B. subtilis* probe, and 4.33×10^3 copies of *B. subtilis* genomic DNA. Cycling conditions were a 10 min enzyme activation step at 95°C, followed by 45–50 cycles of 95°C for 30 s and 60°C for 1 min. For all primer and probe combinations, a no template control was also included. Fluorescence intensities were background corrected and referenced to the ROX reference dye signal.

Examination of Potentially Cross-Reactive Species

A list of potentially cross-reactive bacterial species was generated from the product inserts of manufacturers with commercially available rapid GAS detection kits. The list of potential cross-reactive species included 28 bacteria that are either commonly found in the human mouth or throat or that cause symptoms that could be clinically similar to those associated with GAS or SDSE. The complete list examined and corresponding results can be found in Table 1. All specimens from ATCC (Manassas, VA) were resuspended in 700 μ l Tris buffer, pH 8. Specimens from Microbiologics Inc. (St. Cloud, MN) were in KWIK-STIKTM format

Table 1 Organisms tested for cross-reactivity

Organism	Catalog no. ^a	Organism	Catalog no.
<i>Streptococcus agalactiae</i>	12386	<i>Proteus vulgaris</i>	33420
<i>Streptococcus</i> (group F)	12392	<i>Pseudomonas aeruginosa</i>	10145
<i>Streptococcus pneumoniae</i>	6303	<i>Haemophilus parahaemolyticus</i>	0377P
<i>Streptococcus salivarius</i>	13419	<i>Haemophilus influenzae</i>	0376P
<i>Streptococcus sanguinis</i>	10556	<i>Bordetella pertussis</i>	8467
<i>Streptococcus mitis</i>	0423P	<i>Klebsiella pneumoniae</i>	13883
<i>Streptococcus mutans</i>	700610D ^b	<i>Moraxella catarrhalis</i>	25238
<i>Staphylococcus aureus</i>	12600	<i>Candida albicans</i>	14053
<i>Staphylococcus epidermis</i>	14990	<i>Corynebacterium diphtheriae</i>	11913
<i>Neisseria gonorrhoeae</i>	0375P	<i>Serratia marcescens</i>	0354P
<i>Neisseria lactamica</i>	23970	<i>Yersinia enterocolitica</i>	49397
<i>Neisseria meningitidis</i>	13090	<i>Escherichia coli</i>	700926D ^b
<i>Neisseria sicca</i>	9913	<i>Enterococcus casseliflavus</i>	0117P
<i>Staphylococcus saprophyticus</i>	49907	<i>Enterococcus faecalis</i>	0497P

^a Catalog numbers starting with “0” and ending in “P” are Microbiologics Inc. (St. Cloud, MN). All others are ATCC (Manassas, VA)

^b Specimens with “D” suffix on catalog number were purchased as genomic DNA and were not extracted but used as received

(see www.microbiologics.com). After each pellet was resuspended, the solution was transferred from the plastic assembly into a 1.5 ml microcentrifuge tube. All specimens were then extracted using the Bacterial XpressTM nucleic acid extraction kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. Both real-time PCR assays described earlier were performed using the extracts from each of the potentially cross-reactive organisms as template.

Analytical Sensitivity Comparison to a Rapid Antigen Test

The relative analytical sensitivities of the developed real-time PCR assays were compared to those achieved with a commercially available rapid immunoassay. For these studies, the QuickVueTM Dipstick Strep A rapid immunoassay was graciously provided by Quidel Corporation (San Diego, CA). A large number of colonies were extracted from a 5% sheep’s blood agar plate that was positive for GAS using two sterile rayon swabs supplied with the rapid test kit. The manufacturer’s recommended protocol was followed to release the bacteria from the swab into 780 μ l of 1:1 extraction reagents A and B. Serial 1:10 dilutions were then prepared, using 1:1 extraction reagents A and B as the diluent. Each dilution was divided into two equal aliquots and placed in the small test tubes supplied with the rapid test. The rapid antigen test was immediately performed according to the manufacturer’s instructions on one aliquot of each of the dilution series, and the positive and negative results were recorded for the dilutions. From the other set of aliquots, the nucleic acid was extracted as

described earlier, and the extracts were used as template in real-time PCR reactions for both gene targets using the same conditions described earlier. A negative control was included for each assay. Standard curves were also generated for each assay using GAS genomic DNA of known copy number to enable quantification of the real-time results, with the *B. subtilis* positive control system also included. The copy number present in the GAS standards ranged from $\sim 10^7$ to 10^0 , and was calculated from the mass of gDNA used and the size of the GAS genome (1.85×10^6 base pairs), assuming an average molecular weight of 660 g/mole per base pair of DNA, and one copy of each of the gene targets per genome. The thermal cycling conditions were a 10 min 95°C enzyme activation step, followed by 50 cycles of 95°C for 30 s and 60° for 1 min.

Microarray Development

The hydrolysis probes for the GAS gene target, GAS/SDSE co-detection gene target, and internal control used for real-time PCR were converted into microarray capture sequences by slightly changing the sequence length and adding a 5'-amino-C6 modification. In addition, a negative control oligo and a positive control/spatial marker oligo were included on the array. Capture sequences were ordered desalted from Sigma-Genosys (St. Louis, MO) with a 5'-amino-C6 modification to enable covalent attachment to aldehyde-functionalized glass slides. Capture sequences were spotted at 10 μ M in triplicate from a buffer containing $3 \times$ SSC, 50 mM Na₃PO₄, pH 7.5, and 0.005% N-lauroylsarcosine sodium salt using a QArray Mini

(Genetix, Hampshire, UK) onto aldehyde-functionalized glass slides (CEL Associates, Pearland, TX). A relative humidity of 60% was maintained during spotting, and a High Precision 300 μm solid core pin (Genetix, Hampshire, UK) was used with a stamping time of 30 ms and a pitch of 900 μm . Slides were humidified overnight post-spotting and subsequently stored in a dark slide box at room temperature until used. Prior to hybridization, excess spotting reagents were removed with a 5 min water wash on an orbital shaker.

Microarray Hybridization and Detection

Both traditional fluorescence and the Non-Enzymatic Signal Amplification method (NESATM, InDevR Inc., Boulder, CO) [33] were used as detection methods for the low-density microarray developed. The PCR products for hybridization to the microarray were generated using the same primer sequences utilized for real-time PCR with a 5'-biotin modification on the forward primer and a 5'-phosphate modification on the reverse primer. The gene targets and internal control primers were multiplexed in order to generate PCR products for hybridization to the array. PCR conditions were 3 units Tfi polymerase (Invitrogen Corp., Carlsbad, CA), 1 \times reaction buffer, 10 mM in each dNTP, 200 nM *B. subtilis* primers, 600 nM GAS target gene primers, 600 nM GAS/SDSE gene target primers, 50 mM MgCl₂, and 4.33×10^3 copies of *B. subtilis* gDNA in a 30 μl reaction volume. Cycling was performed in an ABI 9800 Thermal Cycler (Applied Biosystems Inc., Foster City, CA) using a 94°C initial melt for 30 s, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Digestion of the phosphorylated strand of PCR product by lambda exonuclease was used to enable hybridization of single-stranded DNA to the array [34]. PCR products (10 μl) were combined with 1 μl lambda exonuclease (Epicentre Biotechnologies, Madison, WI) and 1.2 μl of 10 \times supplied reaction buffer. The reaction was heated at 37°C for 1 min and the products subsequently added to 20 μl of InDevR hybridization buffer and brought to a total hybridization volume of 40 μl with water. The hybridization mixture was delivered into a silicone hybridization well surrounding the array. The slide was placed in a light-protected humidity chamber and allowed to hybridize for 30 min. After hybridization and washing, the array was labeled with a fluorescent streptavidin conjugate for 5 min in labeling buffer (InDevR Inc., Boulder, CO), and excess label was removed with a wash in 0.2 \times SSC for 5 min on an orbital shaker. Next, 40 μl of PolyAmpTM monomer mixture was delivered into the hybridization well, and photoactivation was conducted with InDevR's IntelliChipTM Reader [33]. After photoactivation, excess

monomer solution was removed with water, and the resulting polymer spots were stained for 2 min using an aqueous solution of 12 mM Rose Bengal and subsequently imaged.

Results and Discussion

Real-Time PCR

Assay Development and Optimization

Figure 1 shows example 4% agarose gel results for the three designed primer pairs indicating amplification of the correct size amplicon in each case. For the GAS/SDSE co-detection and GAS only gene targets shown in lanes 2 and 3, respectively, GAS gDNA (ATCC #700294D-5) was used as template. Once it was determined that the primers functioned as intended, the concentration of the internal control template, primers, and probes for both species were optimized to ensure reliable amplification of the internal control template without sacrificing amplification of the target species. Although they have not been in this study, all three primer sets could feasibly be multiplexed into a single assay.

Figure 2 shows amplification curves for both optimized assays for three different standard specimens. For the GAS-positive specimen shown in Fig. 2a, both gene targets amplified along with the internal control for each assay as

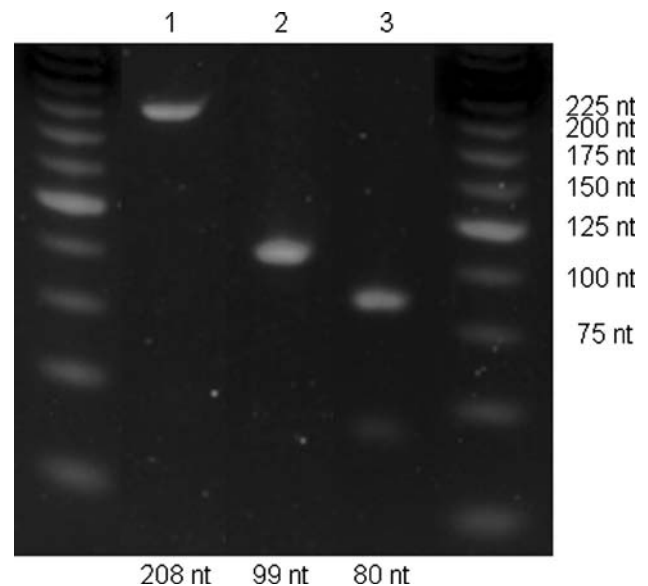


Fig. 1 The 4% agarose gel results. Lane 1 shows *B. subtilis* internal control, lane 2 GAS/SDSE co-detection target, and lane 3 GAS only target. Left and rightmost lanes are 25-bp ladder with lengths shown at right. Amplicon lengths for the three targets are shown at the bottom

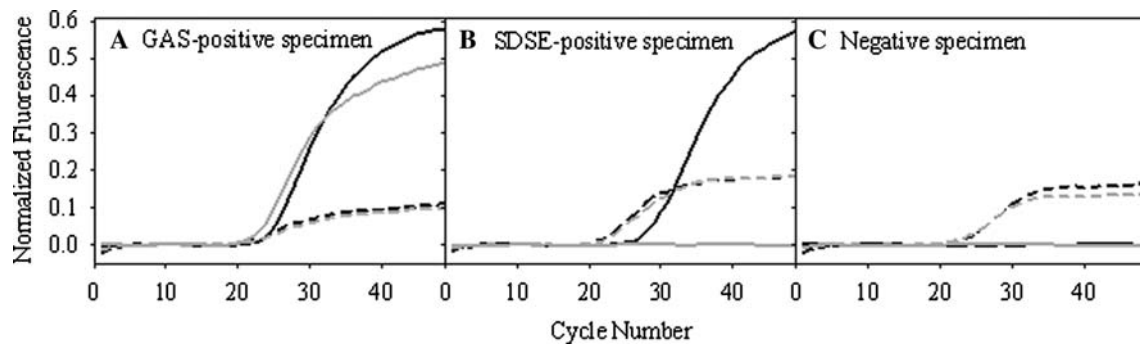


Fig. 2 Typical amplification curves for GAS gene target and GAS/SDSE co-detection gene target with incorporation of exogenous internal control. *Black lines* indicate GAS/SDSE co-detection gene target reaction (*solid line* is gene target, *dashed line* is internal control), and *grey lines* indicate GAS only gene target reaction (again, *solid line* is gene target and *dashed line* is the internal control). **a** A

GAS-positive specimen for which both targets and both internal controls are amplified. **b** SDSE-positive specimen for which the GAS/SDSE co-detection gene target and internal control are amplified. As expected, only the internal control amplified in the GAS only gene target reaction. **c** A negative specimen shows only the amplification of the internal control in both reactions

expected. In Fig. 2b, as expected, an SDSE-positive specimen showed amplification of the GAS/SDSE co-detection gene target (and its accompanying internal control), but showed amplification of only the internal control for the GAS only assay. Lastly, in Fig. 2c, a negative specimen showed only amplification of the internal control for both assays, again indicating the absence of either target organism but no PCR inhibition or failure.

Both real-time PCR assays were examined for cross-reactivity with all of the bacterial organisms listed in Table 1. This list was compiled from the product inserts of several manufacturers offering rapid antigen tests for GAS, and includes other bacteria that are either commensal in the human mouth or throat, or that may cause symptoms that could be confused with those from GAS infection. Neither assay showed cross-reactivity with any of the organisms in Table 1, as evidenced by a lack of amplification during real-time PCR (no discernable C_t value, data not shown).

A recent draft guidance document from the FDA concerning recommendations for in vitro diagnostic tests for influenza (including molecular-based tests) details that potentially cross-reactive bacterial organisms should be tested at a level of 10^6 CFU/ml [35]. For two of the bacteria tested, the starting concentrations were directly calculated. The *Streptococcus mutans* and *Escherichia coli* specimens were received as quantified, purified genomic DNA (no extraction required) and were used in the real-time PCR assays at 3.6×10^8 and 1.6×10^8 genome copies/ml, respectively. If we assume that one bacterium represents 1 CFU, and that there is 1 genome copy/bacterium, the values for *S. mutans* and *E. coli* are more than 2 orders of magnitude higher than the 10^6 CFU/ml recommendation.

All of the other bacteria in Table 1 were received as lyophilized culturable material, and the starting concentrations after extraction were not known. In order to

estimate the starting concentrations of bacteria received as lyophilized culturable material, we compared the amplification of a GAS strain received in the same manner (ATCC #49399) to a standard curve of GAS gDNA. The copy number of the extracted GAS specimen was found to be 1.2×10^{11} copies/ml. Considering that the potentially cross-reactive organisms were received and processed in the same manner, they are also likely well above the 10^6 CFU/ml limit recommended by the FDA.

Comparison of Analytical Sensitivities of Rapid Immunoassay and Real-Time PCR

Figure 3 details the results for the two real-time PCR assays alongside a GAS standard curve. Figure 3a shows the GAS only gene target, and Fig. 3b shows the GAS/SDSE co-detection gene target. In both (a) and (b), the filled circles are the GAS standard curve, and the open circles are the serial dilutions of the cultured GAS specimen prepared as discussed in the “Materials and methods” section. Any dilution that did not have a discernable C_t value is not shown (i.e. the dilutions in which the copy number would be zero). The standard curves were linear over ~ 7 orders of magnitude (R^2 values of 0.9994 and 0.9997 in Fig. 3a and b, respectively), and were used to determine the estimated starting copy numbers in the cultured specimen dilutions. These data are shown in tabular form alongside the RIA results in Table 2, indicating that the real-time PCR assays detect GAS at copy numbers approximately two orders of magnitude below the limit of detection of the rapid antigen test.

Analysis of Clinical Specimens

All real-time PCR assays showing amplification were considered to be a positive result as long as the

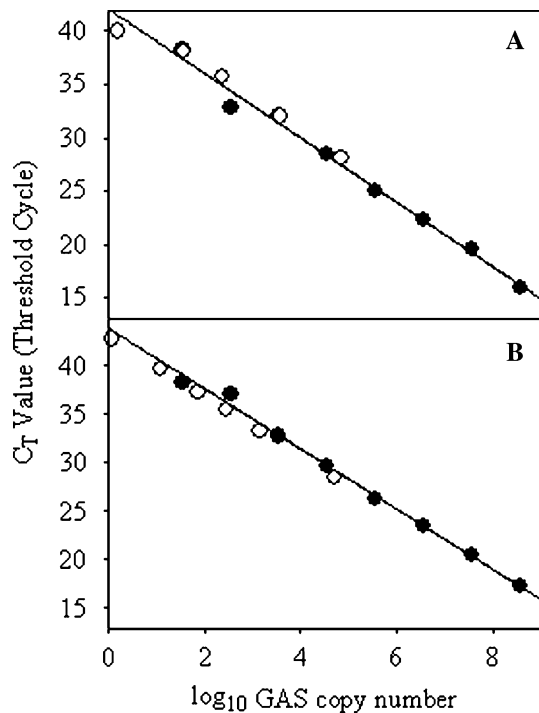


Fig. 3 Real-time PCR results using dilutions of a cultured GAS specimen alongside a GAS standard curve for analytical sensitivity comparison. **a** GAS only gene target. **b** GAS/SDSE co-detection gene target. In both (a) and (b), filled circles are standard curve and open circles are cultured specimen dilutions

Table 2 Analytical sensitivity comparison between real-time PCR and rapid immunoassay for GAS detection

Specimen dilution	RIA result ^b	RT-PCR result ^b	Estimated copy number
<i>GAS only gene target</i>			
[x] ^a	(+)	(+)	6.94E+04
[x]E-01	(+)	(+)	3.79E+03
[x]E-02	(+)	(+)	2.26E+02
[x]E-03	(-)	(+)	3.78E+01
[x]E-04	(-)	(+)	1.57E+00
[x]E-05	(-)	(-)	<1
<i>GAS/SDSE co-detection gene target</i>			
[x]	(+)	(+)	5.13E+04
[x]E-01	(+)	(+)	1.43E+03
[x]E-02	(+)	(+)	2.87E+02
[x]E-03	(-)	(+)	7.39E+01
[x]E-04	(-)	(+)	1.19E+01
[x]E-05	(-)	(+)	1.16E+00
[x]E-06	(-)	(-)	<1

^a The subsequent entries (e.g. '[x]E-01', '[x]E-02') are 10-fold dilutions of the previous row

^b (+) denotes the result was positive for the method shown, and (-) denotes the result was negative

accompanying no template control did not show any amplification through the end of the 50 cycle experiment. Only 3 clinical specimens tested had threshold cycles (C_T) as high as ~ 40 , but in these cases both assays showed amplification, lending additional confidence to their assignment as positives. The four possible combinations of real-time PCR results for the two assays and their interpretation are shown in the first three columns of Table 3. Because GAS contains both gene targets, a positive result for both independent assays was considered a positive GAS identification. A positive GAS/SDSE co-detection gene target assay result combined with a negative GAS only gene target assay was considered a positive identification for SDSE. The specimen was considered "negative" when both assays were negative. The fourth combination in which the assay for the GAS/SDSE co-detection gene target is negative result but the GAS only target is positive should not occur, as both targets should be present for all GAS strains. As expected, this result was not obtained for any of the 96 specimens tested.

Although we are confident that real-time PCR results from these two gene targets can confirm the presence of SDSE, a more complete biochemical characterization to confirm the presence of SDSE for SDSE-positive specimens was not available. Therefore, for the 96 clinical specimens examined here, the real-time PCR results were analyzed only in terms of their ability to detect the presence or absence of GAS. The possible outcomes are shown in the righthand column of Table 3, indicating which situations correspond to a false positive or false negative result using culture as the gold standard method.

A comparison between culture and real-time PCR results for the 96 clinical specimens tested can be found in Table 4. A checkmark in the righthand column indicates agreement between the two methods. Forty-four specimens were positive for GAS by both culture and real-time PCR, and fifteen specimens were negative by both methods. Specimens for which the culture result was positive but produced a 'not A' identification produced two different real-time PCR results: (a) nine specimens (starting with specimen 2 on the bottom left of Table 4) were SDSE-positive results by real-time PCR. As SDSE is a β -hemolytic streptococcal species that is *not* GAS and produces a culture result of 'not A', this result was considered a match between the culture and PCR results for these specimens. (b) Twenty-four specimens (starting with specimen 1 in the top right of Table 4) produced negative results for both real-time PCR assays. We hypothesized that these specimens likely contained other 'not A' β -hemolytic streptococci often found in the human throat such as group B or other species expressing the group C or G antigen. Although not available for every specimen, a matched culture plate was available for eighteen specimens in this

Table 3 GAS detection methods comparison key

	Dual target	GAS only target	PCR result	Culture result	Comparison of ability to identify 'A' or 'not A'
	(+)	(+)	GAS	'A'	TP
	(+)	(+)	GAS	'Not A'	FP
	(+)	(+)	GAS	(-)	FP
	(+)	(-)	SDSE	'A'	FN
	(+)	(-)	SDSE	'Not A'	TP
	(+)	(-)	SDSE	(-)	TP
	(-)	(-)	(-)	'A'	FN
	(-)	(-)	(-)	'Not A'	TP
<i>TP</i> true positive, <i>TN</i> true negative, <i>FP</i> false positive, <i>FN</i> false negative	(-)	(-)	(-)	(-)	TN
	(-)	(+)	Invalid result; repeat analysis		

set that were 'not A' by culture. Latex agglutination for groups A, B, C, F, and G was performed. Consistent with our hypothesis, 10 of these tested positive for Group B strep, 3 for group G, 3 for group C, 1 for group F, and 1 specimen did not agglutinate any of the groups tested.

Only 4 specimens produced discrepant results between culture and real-time PCR (specimen numbers 81, 65, 36, and 44 in Table 4). All four of these specimens were identified as GAS by culture and as 'not A' by real-time PCR. Three of the four specimens produced negative real-time PCR results for both gene targets. One specimen produced a positive result for the GAS/SDSE co-detection gene target and a negative result for the GAS only gene target, denoting the presence of SDSE. One possible reason for this discrepancy is that the developed real-time PCR assays are less sensitive than culture. Considering the number of recent reports that have concluded PCR-based approaches are more sensitive than culture for detection of both viral and bacterial pathogens [24, 36], and in light of our analytical sensitivity results for these assays indicating positive real-time PCR results down to extremely low copy number, we do not believe this is the case. However, it is important to note that the throat swabs received for real-time PCR analysis had already been used to initiate culture. It is reasonable to conclude that there were cases where the "second hand" swab contained a low enough number of bacteria to give a negative result by real-time PCR. Another possible explanation is that the PCR "false negative" results are actually false positive culture results. Culture false positives for GAS occur due to members of the commensal *Streptococcus anginosus* family found in the human mouth and throat that are β -hemolytic and possess the group A antigen [37]. Also, if a culture plate was initially misinterpreted, other Strep bacteria possessing the group A antigen could produce a positive latex agglutination result. Likewise, if the bacterial growth was mixed, a second species with the group A antigen could potentially cause an interfering latex agglutination result.

Conversion to a Low-Density Microarray Format

Although real-time PCR assays may currently be the most common format for molecular-based pathogen testing, capital equipment costs can be prohibitive for smaller laboratories. As an alternative to more expensive fluorescence-based molecular techniques, we developed an alternative non-enzymatic signal amplification method (NESATM) that enables low-cost visual detection for low-density microarrays. Briefly, this colorimetric "on chip" signal amplification method is based on photo-induced polymerization of a labeled, hybridized target, and results in the formation of a solid polymer only in locations where the labeled target is bound to the substrate [33]. The real-time PCR assays were easily converted to function in a low-density microarray format, and the entire process (from specimen extraction through detection) was performed in less than 4 h. Figure 4a shows a digital image taken with an inexpensive reader (<\$5K) after a GAS-positive clinical specimen was hybridized to the low-density microarray and the NESATM protocol was performed. Figure 4b shows a schematic of the microarray. The positive control is present along the top and left for spatial registry and as a hybridization control (filled circles). The other rows each represent a capture sequence spotted in triplicate (open circles). In Fig. 4a, both the positive control and internal control sequences showed positive hybridization (dark spots), indicating no PCR inhibition or failure. Also, both the GAS only gene target and the GAS/SDSE co-detection gene target were positive. As expected, the negative control showed no signal, as it is a non-GAS or SDSE capture sequence and is intended to check for non-specific hybridization.

We introduced a pair of hydrolysis probe-based real-time PCR assays that enable simultaneous detection of *S. pyogenes* (group A strep or GAS) and *S. dysgalactiae* subsp. *equisimilis* (SDSE). Although the need for accurate diagnosis of GAS is well-understood, SDSE is a secondary

Table 4 Results for 96 clinical specimens comparing real-time PCR assays for GAS detection to standard culture

Number	Real-time PCR ^a			Culture result		Number	Real-time PCR ^a			Culture result	
	Dual target ^b	GAS target ^c	Result				Dual target ^b	GAS target ^c	Result		
16	(+)	(+)	GAS	4+ A	✓ ^d	14	(+)	(-)	SDSE	3+ not A	✓
34	(+)	(+)	GAS	4+ A	✓	15	(+)	(-)	SDSE	3+ not A	✓
35	(+)	(+)	GAS	4+ A	✓	18	(+)	(-)	SDSE	2+ not A	✓
37	(+)	(+)	GAS	4+ A	✓	52	(+)	(-)	SDSE	1+ not A	✓
68	(+)	(+)	GAS	4+ A	✓	60	(+)	(-)	SDSE	1+ not A	✓
73	(+)	(+)	GAS	4+ A	✓	1	(-)	(-)	(-)	4+ not A	✓
75	(+)	(+)	GAS	4+ A	✓	6	(-)	(-)	(-)	4+ not A	✓
76	(+)	(+)	GAS	4+ A	✓	8	(-)	(-)	(-)	4+ not A	✓
26	(+)	(+)	GAS	3+ A	✓	45	(-)	(-)	(-)	4+ not A	✓
30	(+)	(+)	GAS	3+ A	✓	46	(-)	(-)	(-)	4+ not A	✓
38	(+)	(+)	GAS	3+ A	✓	3	(-)	(-)	(-)	3+ not A	✓
67	(+)	(+)	GAS	3+ A	✓	9	(-)	(-)	(-)	3+ not A	✓
77	(+)	(+)	GAS	3+ A	✓	41	(-)	(-)	(-)	3+ not A	✓
86	(+)	(+)	GAS	3+ A	✓	49	(-)	(-)	(-)	3+ not A	✓
29	(+)	(+)	GAS	2+ A	✓	56	(-)	(-)	(-)	3+ not A	✓
39	(+)	(+)	GAS	2+ A	✓	57	(-)	(-)	(-)	3+ not A	✓
69	(+)	(+)	GAS	2+ A	✓	17	(-)	(-)	(-)	2+ not A	✓
72	(+)	(+)	GAS	2+ A	✓	19	(-)	(-)	(-)	2+ not A	✓
78	(+)	(+)	GAS	2+ A	✓	23	(-)	(-)	(-)	2+ not A	✓
84	(+)	(+)	GAS	2+ A	✓	31	(-)	(-)	(-)	2+ not A	✓
85	(+)	(+)	GAS	2+ A	✓	40	(-)	(-)	(-)	2+ not A	✓
91	(+)	(+)	GAS	2+ A	✓	43	(-)	(-)	(-)	2+ not A	✓
94	(+)	(+)	GAS	2+ A	✓	47	(-)	(-)	(-)	2+ not A	✓
95	(+)	(+)	GAS	2+ A	✓	63	(-)	(-)	(-)	2+ not A	✓
96	(+)	(+)	GAS	2+ A	✓	21	(-)	(-)	(-)	1+ not A	✓
20	(+)	(+)	GAS	1+ A	✓	24	(-)	(-)	(-)	1+ not A	✓
27	(+)	(+)	GAS	1+ A	✓	50	(-)	(-)	(-)	1+ not A	✓
80	(+)	(+)	GAS	1+ A	✓	48	(-)	(-)	(-)	Rare not A	✓
82	(+)	(+)	GAS	1+ A	✓	51	(-)	(-)	(-)	Rare not A	✓
88	(+)	(+)	GAS	1+ A	✓	10	(-)	(-)	(-)	(-)	✓
90	(+)	(+)	GAS	1+ A	✓	13	(-)	(-)	(-)	(-)	✓
92	(+)	(+)	GAS	1+ A	✓	22	(-)	(-)	(-)	(-)	✓
93	(+)	(+)	GAS	1+ A	✓	25	(-)	(-)	(-)	(-)	✓
7	(+)	(+)	GAS	Rare A	✓	28	(-)	(-)	(-)	(-)	✓
11	(+)	(+)	GAS	Rare A	✓	32	(-)	(-)	(-)	(-)	✓
4	(+)	(+)	GAS	A	✓	33	(-)	(-)	(-)	(-)	✓
5	(+)	(+)	GAS	A	✓	42	(-)	(-)	(-)	(-)	✓
53	(+)	(+)	GAS	A	✓	70	(-)	(-)	(-)	(-)	✓
54	(+)	(+)	GAS	A	✓	71	(-)	(-)	(-)	(-)	✓
55	(+)	(+)	GAS	A	✓	74	(-)	(-)	(-)	(-)	✓
59	(+)	(+)	GAS	A	✓	79	(-)	(-)	(-)	(-)	✓
61	(+)	(+)	GAS	A	✓	83	(-)	(-)	(-)	(-)	✓
62	(+)	(+)	GAS	A	✓	87	(-)	(-)	(-)	(-)	✓
66	(+)	(+)	GAS	A	✓	89	(-)	(-)	(-)	(-)	✓
2	(+)	(-)	SDSE	4+ not A	✓	81	(-)	(-)	(-)	3+ A	FN
58	(+)	(-)	SDSE	4+ not A	✓	65	(-)	(-)	(-)	Rare A	FN

Table 4 continued

Number	Real-time PCR ^a			Culture result		Number	Real-time PCR ^a			Culture result	
	Dual target ^b	GAS target ^c	Result				Dual target ^b	GAS target ^c	Result		
64	(+)	(-)	SDSE	4+ not A	✓	36	(-)	(-)	(-)	2+ A	FN
12	(+)	(-)	SDSE	3+ not A	✓	44	(+)	(-)	SDSE	4+ A	FN

FN false negative

^a Since we do not have definitive positive identification of SDSE by methods other than real-time PCR, the real-time PCR results here were analyzed in terms of the ability to identify a specimen as 'A' or 'not A'

^b This gene target produces a positive real-time PCR result for both GAS and SDSE

^c This gene target produces a positive result only for GAS

^d A checkmark in this column denotes either a true positive or true negative result (and therefore, agreement between the two methods)

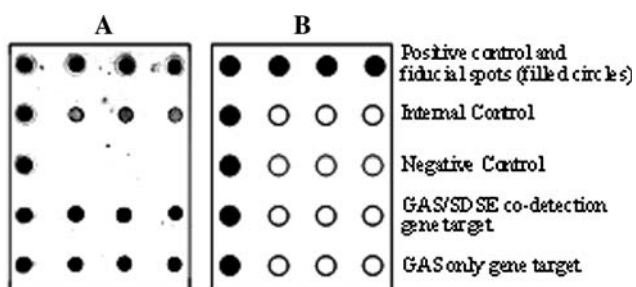


Fig. 4 **a** Digital transmission image of low-density microarray after extracted material from a GAS-positive specimen was hybridized to the array and processed as described in the “Materials and methods” section. *Dark spots* indicate positive signal (decreased transmission). **b** Schematic drawing of the array layout with individual sequences on the array listed at right

pathogen whose clinical importance has not been completely elucidated. A real-time PCR assay to detect SDSE and differentiate it from GAS should enable more detailed investigations of its prevalence and clinical importance, and is an improvement over current presumptive identification methods. The assays showed over 2 orders of magnitude improved analytical sensitivity compared to a rapid immunoassay, and demonstrated clinical sensitivity of 91.7% and clinical specificity of 100% compared to standard culture in an analysis of 96 throat swabs. These analyses could be performed to provide a same day result in many larger and appropriately equipped reference and hospital laboratories.

To address the need for molecular diagnostic tests in more resource-limited settings where real-time PCR may not be available, we converted the real-time PCR assays into a low-density microarray format using an inexpensive non-enzymatic signal amplification method. In combination with traditional PCR and a low-cost instrument, this approach has a time to result of less than 4 h from specimen collection.

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