REAL-TIME PCR DETECTION OF GROUP A STREPTOCOCCUS USING THE 3M INTEGRATED CYCLER

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Abstract

Because Group A Streptococcus (*S. pyogenes*) infections can result in serious sequelae if they are not treated, accurate detection tests are needed. Antigen detection tests are relatively rapid, but they have limited sensitivity; follow-up testing by culture is recommended to reduce the risk of false negative results. Real-time PCR may offer a better balance of performance and turn-around time. Using Simplexa chemistries, we developed a real-time PCR for detecting Group A Streptococcus. Results were compared to standard microbiological methods, and discrepants were resolved using a second PCR and sequencing.
Results from 220 patient samples and 30 cultured organism isolates were compared to standard microbiological methods, and discrepant results were resolved using a second PCR and sequencing. The Simplexa assay showed no cross reactivity with other common pathogens, and microbiological methods only detected normal flora from 10 samples for which PCR methods and sequencing methods were able to detect Group A streptococcus.

Methods

Patient Samples: Throat swabs were collected in liquid Amies or other appropriate commercially available media, and were submitted to Quest Diagnostics (West Hills, CA) for aerobic culture and Group A Streptococcus identification. After deidentifying the samples, they were subjected to a nucleic acid isolation procedure using the Roche MagNA Pure LC instrument and the corresponding Total Nucleic Acid Isolation Kit with the variable elution protocol (to enable elution in 50 uL).

Organism Isolates: See Table 1 for a list of bacteria and viruses. Organisms were obtained from ZeptoMetrix (Buffalo, NY) and from the American Type Culture Collection (Manassas, VA). Bacteria and Candida were diluted to a concentration of 10^6 CFU/mL, and viruses to a concentration of 10^5 TCID50/mL. Diluted samples were then extracted using the Roche MagNA Pure LC instrument and corresponding Total Nucleic Acid Isolation Kit.

Cross-reactivity: A total of 30 diluted organism preparations (from commercial sources) and 220 patient samples were tested with the Simplexa assay, and results were compared to standard microbiological methods. Discrepant samples were repeated in the Simplexa assay. If the Simplexa results repeated as positive, then the samples were tested in a SYBR green PCR, and the amplicon was sequenced.

Simplexa Assay: The Simplexa Group A Streptococcus Real-Time PCR included a primer pair that targeted a well-conserved region of the Group A Streptococcus exotoxin B gene, and was labeled with 6-carboxyfluorescein (FAM). The assay also included an internal extraction and amplification control, with a corresponding primer pair labeled with Quasar 670 (Q670). All data was generated using the Integrated Cycler amplification instrument. Note: the Simplexa assay is in development, is not currently available for sale, and is not FDA cleared. Data is preliminary.

SYBR Green PCR and discrepant analysis: A second PCR was developed for discrepant analysis. The second PCR used SYBR green chemistry, the primers targeted a different region of the GAS exotoxin B gene, and was performed with an ABI 7500. DNA extracts (10 uL) from samples with discrepant results were amplified with 15 uL iTaq™ SYBR® Green Supermix (Bio-Rad) in two stages, 95°C for 15 seconds and then 50 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Amplicons generated from the discrepant samples were then analyzed for their melting temperature, and then sent to SeqXcel (San Diego, CA) for purification and sequencing in both directions using the chain-termination method. DNA extracts from samples with discrepant results were amplified and sequenced.

Methods (Cont.)

Results

Cross-reactivity with spiked organism panel: The Simplexa assay did not show cross-reactivity with any of the organisms listed in Table 1. Furthermore, the results also show that all controls gave correct results (including the internal control, which was amplified in each sample, showing that extraction and amplification processes appeared to be functioning properly).

Table 1. Cross reactivity studies using cultured isolates

Organisms	Real-Time PCR Ct Value	
	Group A Streptococcus	Internal Control
Adenovirus 7A	0	28.2
Bordetella pertussis	0	30.3
Candida albicans	0	30.7
Chlamydophila pneumoniae	0	35.3
Coronavirus 229E	0	29.7
Corynebacterium diphtheriae	0	30.5
Cytomegalovirus	0	29.9
Epstein-Barr Virus	0	29.1
Enterovirus 71	0	29.6
Group B streptococcus	0	29.6
Group C streptococcus	0	30
Group G streptococcus	0	29.5
Haemophilus influenzae	0	30.1
Human metapneumovirus	0	29.4
Influenza A	0	29.2
Influenza B	0	29.1
Klebsiella pneumoniae	0	30.2
Legionella pneumophila	0	30.3
Moraxella catarrhalis	0	30.3
Mycoplasma pneumoniae	0	29.3
Neisseria meningitidis	0	31.2
Parainfluenza Type-1	0	30.5
Parainfluenza Type-2	0	29.8
Parainfluenza Type-3	0	28.8
Pseudomonas aeruginosa	0	29.7
Rhinovirus	0	29.5
Respiratory Syncytial Virus	0	31.2
Staphylococcus aureus	0	33.5
Streptococcus pneumoniae	0	30.2
Streptococcus salivarius	0	30
Positive Control	24.6	30.2
Negative Control	0	30.6

Results (Cont.)

Cross-reactivity with Patient Samples: Organisms were recovered from all 220 patient samples organisms by microbiological methods (normal flora, or other organisms as listed in Table 2). Of the 220 specimens, 37 were determined to be positive for Group A Streptococcus using both microbiological methods and the Simplexa assay. Ten samples that were determined to be negative by microbiological methods were positive by both PCR methods and by sequencing. One of the samples originally identified as positive by the Simplexa assay was determined to be Group G streptococcus positive by microbiological methods; however this sample was negative upon repeat testing, and upon testing with the SYBR green PCR method, suggesting some cross contamination in the initial test setup. A total of 172 samples were determined to be Group A Streptococcus negative by PCR; of these samples, microbiological methods identified non-GAS pathogens in 21 patient samples, and identified normal flora in the other 151 samples. One culture positive sample that was originally negative for Group A Streptococcus by PCR was determined to be positive by both PCR assays and by sequencing on repeat/discrepant analysis (note that upon repeat analysis the Simplexa PCR showed a Ct value of 38, and the SYBR green PCR had a Ct value of 47, indicating that the sample was a very low positive specimen).

Discrepancy Resolution: The 10 Simplexa real-time PCR positive samples that were listed by microbiological methods as being normal flora were determined to be positive by the SYBR Green-based PCR (Table 3). All the positive samples demonstrated a signature melting temperature at approximately 79.5°C as did of the positive control (Fig.1), while the negative samples did not generate signals or melting temperature profiles. The 256 bp amplicons produced by the SYBR green assay were purified and sequenced in both 5' and 3' directions using the dideoxy chain-termination method. Over 200 bases of the amplicon were determined for each sample, and these results confirmed the presence of Group A Streptococcus sequences (which was determined by comparison to available sequences in the GenBank database).

Table 2. Cross reactivity and PCR results compared to microbiological methods.

Microbiological Methods	Simplexa	
	Positive	Negative
Group A Streptococcus	37	1
Beta Hemolytic non-Group A Streptococcus	0	5
Group C Streptococcus	0	5
Group G Streptococcus	1*	5
Non- Group A, C, or G Streptococcus	0	3
Haemophilus influenza	0	1
Staphylococcus aureus	0	1
Normal Flora	10	151
Total	48	172

* Sample was negative upon repeat testing and with the alternate PCR method.

Results (Cont.)

Table 3. Resolution of discrepant samples using alternate PCR and DNA Sequencing.

Sample ID	Simplexa (Repeat)	PCR (SYBR Green)	Sequencing Result
8	+	+	Group A Streptococcus
38	+	+	Group A Streptococcus
61	+	+	Group A Streptococcus
104	+	+	Group A Streptococcus
108	+	+	Group A Streptococcus
143	+	+	Group A Streptococcus
147	+	+	Group A Streptococcus
186	+	+	Group A Streptococcus
190	+	+	Group A Streptococcus
203	+	+	Group A Streptococcus
20*	+	+	Group A Streptococcus
191**	-	-	N/A

* Group A Streptococcus positive by microbiological methods
** Group G streptococcus positive by microbiological methods
All other samples are normal flora by microbiological methods

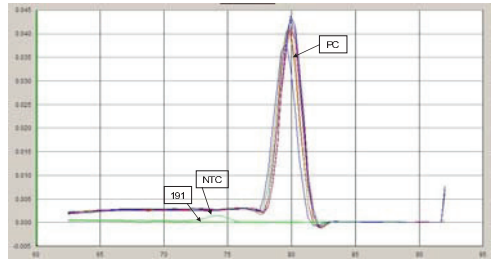

Figure 1. Melting temperature analysis of amplicons produced with the SYBR Green-based PCR.

Conclusions

- The Simplexa assay showed no cross-reactivity with isolates of 30 different human pathogens.
- Standard microbiological methods failed to detect some patient samples containing Group A Streptococcus.

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