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RAPID DETECTION OF GROUP A STREPTOCOCCUS (*Streptococcus pyogenes*) USING THE SIMPLEXA™ GROUP A STREP DIRECT ASSAY

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Modified Abstract

Introduction: Group A streptococcus (*S. pyogenes*) infection can result in serious sequelae if not treated, so a quick and accurate diagnosis is important. Traditional antigen detection tests have limited sensitivity and need additional confirmatory culture testing to reduce the risk of false negative results. Consequently, needed treatment may be delayed. In an effort to provide an alternative to the antigen detection test, we are developing a real-time PCR assay that detects Group A streptococcus directly from throat swabs in approximately 1 hour.

Methods: The Simplexa™ Group A Strep Direct Assay (Simplexa Direct assay) targets the conserved exotoxin B gene of *S. pyogenes*. For each assay, 50 µL of an Eswab® patient throat swab sample and 50 µL of Direct Mix were added to their respective wells on the Direct Amplification disc, followed by amplification in the 3M™ Integrated Cycler. Assay results were compared with culture results (n=105). Discrepant results were resolved by amplifying and sequencing a region of the exotoxin B gene different from that targeted by the Simplexa assay. Analytical limit of detection (LoD), specificity, interference and reproducibility studies were also performed.

Results: Preliminary results showed that, in comparison to culture, positive and negative agreement of the Simplexa Direct assay were 100% and 81.4%, respectively. GAS specimens that were discordant (Simplexa positive and culture negative) were PCR amplified followed by sequencing confirmation. Out of the 16 discordant samples, 15 specimens were confirmed to be Group A Strep. The LoD of the assay was 20 CFU per reaction. No cross-reactivity was observed with 30 common pathogens. No interference was observed from any of the 15 potential interfering substances, including blood, antibiotics, and over-the-counter remedies for sore throat. Reproducibility studies yielded <2.3% total coefficient of variation among replicates.

Conclusions: The Simplexa Direct assay detected positive samples that culture missed, as confirmed by sequencing results. With no requirement for nucleic acid extraction, the Simplexa Direct assay provides a rapid molecular solution that does not compromise detection sensitivity.

Introduction

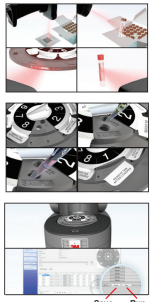
Although a majority of pharyngitis cases are caused by viruses, clinicians often prescribe antibiotics for pharyngitis. The Infectious Disease Society of America guidelines recommend Group A streptococcus testing in appropriate patient populations to determine whether it is the cause of diseases such as pharyngitis. This knowledge can in turn guide appropriate antibiotic use, which helps to prevent post-infection complications such as rheumatic fever, reduce transmission to others, and reduce unnecessary antibiotic use. Group A streptococcus is commonly detected using rapid antigen tests. However, negative antigen test results may require confirmation by culture or other methods due to the lack of sensitivity of these tests. To overcome this need for confirmatory testing, we developed an assay to rapidly and sensitively detect Group A streptococcus. This Simplexa Direct assay requires little user input or training. The chemistry enables direct detection from specimens without nucleic acid extraction to simplify workflow and decrease time to results.

Methods

Patient Sample Preparation: 105 throat swabs were collected from subjects with presumptive Group A Streptococcus using the ESwab Transport System (Becton Dickinson). The samples were de-identified and cultured for the presence of Group A streptococcus.

Simplexa Direct Assay: Sample setup and testing were performed as outlined in Figure 1.

Methods (Cont.)



Step 1: SCAN
- Assay definition using the card provided in the kit. *Only done the first time each lot is used.*
- Reagent barcode on the Simplexa™ Reaction Mix vial.
- Direct Amplification Disc barcode.
- Patient ID.

Step 2: PREPARE
- Lift the foil from the well.
- Pipette Direct Reaction Mix into "R" well.
- Pipette unprocessed patient sample into "SAMPLE" well.
- Re-seal wells with foil and tear off performated edge.

Step 3: RUN
- Place Direct Amplification Disc onto the plate and close the lid.
- Click "Save"; then click "Run."

Figure 1. Simplexa Direct Assay Setup

Discrepant Analysis: A SYBR® Green-based PCR assay was developed to analyze samples giving discrepant results in the Simplexa Direct assay and culture. Specimens were initially heated at 97°C for 8 minutes, and 5 µL of sample was then amplified using iTaq™ SYBR Green SuperMix With ROX (Bio-Rad). The amplicons were then sequenced bi-directionally and the results were compared to available sequences using the Basic Local Alignment Search Tool (BLAST).

Limit of Detection (LoD) Study: Group A streptococcus M1 (ATCC# 700294) and M3 (ATCC # BAA-595) strains (American Type Culture Collection, Manassas, VA) were serially diluted in negative swab matrix, and 24 replicates of dilutions with concentrations at or near the presumptive limit of detection were amplified. The LoD was defined as the lowest concentration at which at least 95% of replicates were detected.

Reproducibility Study: Low-positive (4X LoD) and medium-positive (10X LoD) samples were prepared using the M1 stock in negative swab matrix. The study was conducted in triplicate with 3 operators using 2 instruments for 3 days.

Cross-reactivity Study: This study determined the cross-reactivity with various organisms obtained from ZeptoMetrix (Buffalo, New York) and from American Type Culture Collection (Table 1). Before testing, bacteria were diluted to a concentration of 10^6 CFU/mL and viruses were diluted to 10^5 $TCID_{50}$/mL.

Table 1. Organisms Tested for Cross Reactivity

Organisms	
Adenovirus 1	Human metapneumovirus
Archanobacterium haemolyticum	Influenza A
Bordetella pertussis	Influenza B
Candida albicans	*Legionella pneumophila*
Chlamydia pneumoniae	*Moraxella catarrhalis*, Ne 11
Coronavirus 229E	*Mycoplasma pneumoniae*, strain M129
Corynebacterium diphtheriae	*Neisseria meningitidis*
Cytomegalovirus	Parainfluenza type 1
Epstein Barr Virus	Parainfluenza type 2
Enterovirus 71	Parainfluenza type 3
Streptococcus agalactiae (Group B Strep)	Rhinovirus 1A
Streptococcus equi (Group C Strep)	Respiratory Syncytial Virus
Streptococcus canis (Group G Strep)	*Staphylococcus aureus*, COL (MRSA)
Haemophilus influenzae	*Streptococcus pneumoniae*
HSV-1	*Streptococcus salivarius*

Methods (Cont.)

Interference Study: PCR amplification was performed on a panel of Group A streptococcus low-positive (3X LoD) samples in the presence of potential interfering substances. These substances included blood, mucin, antibiotics, throat lozenges, nasal sprays, cold and flu medications, and pain medications (Table 2).

Table 2. List of Potential Interfering Substances Tested with Assay Results

Potential Interferent	Active Ingredient(s)	Final Concentration	Simplexa Ct
Chloraseptic Sore Throat Spray	Phenol	10% (v/v)	33.8
	Amoxicillin	0.5 mg/ml	34.3
Antibiotics	Erythromycin	1 mg/ml	33.3
	Penicillin	6000 Units/ml	33.4
	Zinc gluconate glycine	0.1 mg/ml	32.0
Sore throat Lozenge	Pectin	1.7 mg/ml	33.7
	Menthol	1.7 mg/ml	33.9
NyQuil	Dextromethorphan hydrobromide	1/200X dilution	33.7
	Doxylamine succinate		
Pain medication	Tylenol (acetaminophen)	1 mg/ml	33.1
	NASIDs (ibuprofen)	0.1 mg/ml	33.8
Afrin Nasal Spray	Oxymetazoline hydrochloride	15% (v/v)	33.7
Neo-Synephrine	Phenylephrine HCl	15% (v/v)	33.5
Mucin	Purified mucin protein	60 ug/mL	33.6
Blood (human)	N/A	2% (v/v)	33.2
Saline Nasal Spray	Sodium chloride with preservatives	15% (v/v)	34.7
Positive Control			33.1
Negative Control			0.0

Results

Comparison of Simplexa Direct Assay with Culture: The results of the Simplexa assay showed 100% positive agreement and 81.4% negative agreement with those of culture (Table 3). Of 105 specimens, 70 were negative in both the Simplexa assay and culture; the remaining 35 samples were positive in the Simplexa assay, but 16 of these were discordant (Simplexa positive and culture negative). Sequencing confirmed that 15 of 16 discrepant samples were Group A streptococcus positive. The remaining specimen could not be amplified by the SYBR Green-based PCR, probably because of a low concentration of Group A streptococcus (Ct was 37.4).

Table 3. Comparison of Simplexa Direct Assay to Culture

		Culture			
		GAS Positive	GAS Negative	Total	% Agreement
Simplexa Group A Strep Direct	GAS Positive	19	16[a]	35	% Positive Agreement 100% (19/19)
	GAS Negative	0	70	70	% Negative Agreement 81.4% (70/86)
	Total	19	86	105	

[a] 15 out of 16 discrepant samples were resolved by sequencing analysis
GAS: Group A Streptococcus

Ct value distributions of both concordant and discordant samples are shown in Figure 2. The values overlap significantly between the 2 groups. Culture detected samples with a Ct range from 20 to 33, but did not detect any sample with Ct ≥34 and missed several samples with Ct between 25 and 32. Samples with relatively high Group A streptococcus concentrations may be overlooked by culture because of the presence of normal pharyngeal flora which concealed Group A streptococcus bacteria.

Results (Cont.)

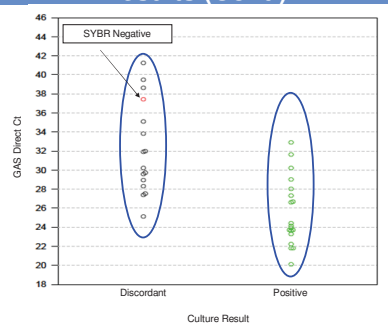


Figure 2. Ct Distribution of Both Concordant and Discordant Samples

Limit of Detection (LoD): As shown in Table 4, the Simplexa Direct assay LoD was 20 CFU per reaction for both M1 and M3 strains.

Reproducibility: The coefficient of variation (CV) values of total variability, including between-instrument, between-operator, between-run, and within-run reproducibility for the low-positive and medium-positive samples, were 1.9% and 2.3%, respectively (data not shown).

Cross-reactivity: The Simplexa Direct assay did not cross-react with any of the organisms listed in Table 1, as evidenced by the lack of amplification (no amplification plots were observed and no Ct values were generated).

Interference: No inhibition of detection was observed with the substances shown in Table 2, which were present at relevant concentrations.

Table 4. Limit of Detection Study

Group A Strep	Cfu/mL	Replicates Detected	Average Ct
M1	2000	22/23*	37.4
M3	2000	24/24	36.9

* 1 of the 24 M1 replicates failed due to lack of internal control detection

Conclusions

- Limit of The Simplexa Group A Strep Direct assay detected more positive samples than culture. Most of the discrepant samples (15/16) were confirmed to be Group A streptococcus positive by sequencing.
- Limit of detection of the Simplexa Direct assay was 20 CFU per reaction.
- Reproducibility of the Simplexa Direct assay was good (CV <2.3%).
- No interference with other substances was observed.
- No cross-reactivity with common pathogens was observed.
- This Simplexa Group A Strep Direct assay is in development, it is not currently available for sale, and is not FDA cleared.