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Evaluation of Simplexa Group A Strep Direct Kit Compared to Hologic Group A Streptococcal Direct Assay for Detection of Group A *Streptococcus* in Throat Swabs

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ABSTRACT Diagnosis of bacterial pharyngitis is confirmed by detection of group A *Streptococcus* (GAS) in patient throat samples. Testing of throat samples has historically relied on culture, but new molecular methods allow much faster test turnaround time (i.e., same day versus 48 to 72 h for culture). Our laboratory uses the Hologic GAS Direct (GASD) assay for screening more than 125,000 throat samples per year. Simplexa GAS Direct is a new real-time quantitative PCR (qPCR) assay that does not require initial DNA extraction. Performance of Simplexa qPCR was compared to GASD. A total of 289 throat swabs were collected from patients attending ambulatory clinics in Calgary, Alberta, Canada. A total of 60 (20.8%) of the samples were initially GAS positive by either method: 54 by both methods, 4 by Simplex qPCR alone, and 2 by GASD alone. An in-house PCR using a unique GAS primer set was used to resolve the 6 discrepant results. Overall, GASD compared to Simplexa qPCR had a sensitivity, specificity, positive predictive value, and negative predictive value of 93.1% versus 100%, 100% versus 100%, 100% versus 100%, and 98.31% versus 100%, respectively. Implementation of Simplexa qPCR in our laboratory setting would cost more but allow the high sample volume to be reported in half the time and save 0.62 medical laboratory technician (MLT) full-time equivalent (FTE). In comparison to culture, the implementation of Simplexa qPCR would save 2.79 medical laboratory assistant (MLA) FTE plus 0.94 MLT FTE. Simplexa qPCR has improved performance and diagnostic efficiency in a high-volume laboratory compared to GASD for GAS detection in throat swabs.

KEYWORDS group A *Streptococcus*, molecular methods, pharyngitis

Streptococcus pyogenes, also known as group A *Streptococcus* (GAS), is a beta-hemolytic bacterium that belongs to Lancefield serogroup A. GAS is the most common bacterial cause of pharyngitis in developed countries, accounting for 15 to 30% of the acute pharyngitis cases in children and 5 to 20% of the cases in adults (1, 2). Although GAS pharyngitis is most commonly diagnosed in school-age children and affects approximately 1 in 10 children per year (2, 3), 15% of young children may also be asymptomatic pharyngeal GAS carriers (4). Various respiratory viruses, however, cause most cases of non-GAS pharyngitis, including adenovirus, influenza virus, parainfluenza virus, rhinovirus, and respiratory syncytial virus (5, 6). Although GAS pharyngitis is usually self-limiting, rapid and accurate detection is important, as early treatment with appropriate antibiotics reduces symptom severity and duration, decreases transmission of the organism, and reduces the risk of glomerulonephritis and rheumatic fever (7–9). Because most pharyngitis is viral in origin, accurate diagnosis can reduce the unnecessary use of antibiotics and potential development of antibiotic resistance (10, 11). Distinguishing bacterial from viral pharyngitis using clinical signs alone is

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unreliable; it has been documented that physicians miss up to 50% of GAS pharyngitis cases and identify 20 to 40% of non-GAS sore throat cases as requiring antibiotics (2, 12, 13). Reducing the burden of GAS pharyngitis among children is also economically important as it is estimated that between $224 and $539 million is expended annually in the United States because of this disease (14).

Direct culture of throat swabs remains the "gold standard" for laboratory detection of GAS because of its high sensitivity (90 to 95%) and ability to detect the presence of other much less common bacterial causes of pharyngitis (e.g., ~2% of cases may be due to large-colony group C *Streptococcus* and *Arcanobacterium haemolyticum*), use of this method results in delayed (24 to 72 h) result reporting (11, 15, 16). The existing guideline in our province recommends early detection of GAS using a culture-based method or equivalent (17). Our laboratory has used the Hologic GAS Direct (GASD) test for more than a decade because of its documented equivalence to culture while allowing for more rapid reporting of results (i.e., 24 h after specimen receipt versus 24 to 48 h for culture) (18, 19). It was of interest, however, to investigate replacement of our current method with a commercial real-time PCR test that potentially would offer improved detection performance and increased efficiency for analysis of the high volume of throat samples submitted to our laboratory (i.e., >125,000 per year in 2015) so that same-day reporting of results was maintained. Several commercial real-time PCR assays have recently been licensed for detection of *S. pyogenes* from throat swabs. Although these assays provide a sensitivity and specificity comparable to that of culture, DNA extraction is commonly required prior to performing quantitative PCR (qPCR) (20–22). Both the Solana GAS (Quidel, San Diego, CA) and the cobas Liat GAS (Roche Molecular Diagnostics, Branchburg, NJ) assay systems were designed to either perform a small number of tests per run or a single test at point of care, respectively. The Simplexa GAS Direct qPCR assay was therefore evaluated because it was recently licensed by the Health Protection Branch (HPB) in Canada, does not require sample extraction, and is conducive to high-volume laboratory testing. Currently there is one reported evaluation of the Simplexa qPCR assay that was recently performed by the manufacturer (22). The performance of GASD was clinically and economically compared to the Simplexa Direct GAS real-time assay in our regional laboratory setting.

MATERIALS AND METHODS

All patient tests were performed by the centralized regional microbiology laboratory at Calgary Laboratory Services (CLS). CLS provides services to an urban and rural population of ~1.5 million people in Calgary and surrounding areas in southern Alberta. CLS routinely uses the Hologic GASD hybridization assay (GASD) (Hologic, San Diego, CA) to detect GAS from throat swabs collected from patients with suspected bacterial pharyngitis. The Hologic GASD DNA probe assay is a nucleic acid hybridization test that uses a single-stranded DNA probe with a chemiluminescent label that is complementary to the rRNA of the target organism. After the rRNA is released using a lytic reagent, the labeled DNA probe combines with the target organism's rRNA to form a stable DNA-RNA hybrid. The selection reagent differentiates nonhybridized from hybridized probe. The labeled DNA-RNA hybrids are measured in a Leader 450i luminometer (Hologic). A positive result is a luminometer reading greater than or equal to the cutoff. A value below this cutoff is a negative result. Throat swabs are only cultured on patients with a reported penicillin allergy or those with treatment failure. Children's samples that give a low positive reading at the GASD cutoff (4,500 to 10,000 relative light units [RLU]) are also cultured to confirm the result.

The performance of GASD was compared with that of the Simplexa GAS Direct real-time PCR assay (Focus Diagnostics [DiaSorin Group], Cypress, CA) on a total of 289 individual patient's throat swabs that were sequentially retrieved from continuously submitted clinical samples. Throat swabs were physician collected using Copan swabs in liquid Amies transport tubes (Copan Diagnostics, Inc., Murietta, CA) and transported within 4 to 6 h to the laboratory. All throat swabs were initially processed and tested using the GASD assay strictly according to the manufacturer's instructions. During processing for GASD, the original throat swab was placed in 300 μl of lysis reagent in a polypropylene tube, which was then heated in a dry heat bath for 10 min at 95 $\pm$ 3°C. The tubes were then cooled for 5 min before thoroughly expressing each swab against the side of the tube to remove as much liquid as possible before discarding the swab. A 50-μl aliquot was then removed and transferred to a clean, labeled polypropylene tube for testing by GASD. The remaining swab lysate was refrigerated overnight before Simplexa qPCR testing was performed. A sterile flocked swab (eSwab) (Copan Diagnostics, Inc., Murrieta, CA) was then placed into the original transport medium and vigorously rotated around in the sponge containing plain Amies transport medium. The inoculated swab was then transferred into the eSwab liquid transport medium and vigorously vortexed before processing and performing the Simplexa GAS qPCR assay thereafter according to the manufacturer's instructions. A Janus G3 automated liquid handling system (Perkin-

TABLE 1 Performance of Simplexa GAS Direct qPCR compared to the Hologic GAS Direct hybridization assay

Performance parameter	Result for[a]: Simplexa GAS qPCR No. positive/ tested (%)	Simplexa GAS qPCR 95% CI	GASD No. positive/ tested (%)	GASD 95% CI
Sensitivity	60/60 (100)	0.940–1.0	54/58 (93.1)	0.825–0.977
Specificity	229/229 (100)	0.984–1.0	231/231 (100)	0.979–1.0
PPV	58/58 (100)	0.942–1.0	54/54 (100)	0.917–1.0
NPV	231/231 (100)	0.986–1.0	231/235 (98.3)	0.954–0.995

[a]True positive represents GAS confirmed by both molecular methods or either molecular method and the in-house PCR. GASD, Hologic GAS Direct hybridization assay; CI, confidence interval.

Elmer, Woodbridge, Ontario, Canada) was used to pipette 95 samples plus 1 negative control (uninoculated eSwab liquid transport medium) and the manufacturer's PCR master mix and internal control into the Simplexa 96-well direct amplification disc (DAD [described below]) prior to qPCR testing.

The Simplexa qPCR Group A Strep Direct assay (Focus Diagnostics, Cypress, CA [distributed in Canada by Bionuclear Canada, Oakville, Ontario]) has been approved by the Health Protection Branch (HPB), Canada, to perform direct amplification and qualitative detection of bacterial DNA from throat swabs without nucleic acid extraction (22). The system consists of the Simplexa qPCR Group A Strep Direct assay (HPB license no. 85109), the 3M Integrated cycler (with Integrated Cycler Studio software; HPB license 84906), the Direct Amplification 96-well disc (DAD), and associated accessories. In the Simplexa qPCR Group A Strep Direct assay, bifunctional fluorescent probes and primers are used together with corresponding reverse primers to amplify GAS bacterial DNA and the internal control (DNA IC). The assay targets a conserved region of GAS (pyrogenic exotoxin B gene) to identify this bacterium in the specimen. The DNA IC is used to detect PCR failure and/or inhibition. Each Simplexa qPCR Group A Strep Direct kit contains sufficient reagents for 24 reactions.

All discrepant results between GASD and the Simplexa qPCR assay were resolved by performing an in-house qPCR that used a unique GAS primer set targeting the *S. pyogenes spy* gene as previously reported (21, 23). Each qPCR was done in a 25-μl total reaction volume containing 5 μl template DNA plus 20 μl reaction mixture, 0.75 μl of 10 μM *spy* F/R primers and 10 μM ZEN *spy* probe labeled with 6-carboxyfluorescein–Iowa Black fluorescent quencher (FAM-IABFQ), 5.75 μl sterile water, and 12.5 μl TaqMan Universal Mastermix II, with uracil *N*-glycosylase (UNG; Thermo Fisher Scientific, Waltham, MA). qPCR was performed through 45 cycles of 95°C with 15 s of denaturation followed by 60°C for 1 min for annealing and extension. All in-house qPCRs were performed using a Vii7A real-time PCR system (Applied Biosystems, Inc., Thermo Fisher Scientific, Foster City, CA).

Data were entered into a Microsoft Excel (2010) spreadsheet (Microsoft Corp., Seattle, WA) and analyzed using Analyze-it software (Microsoft Corp.). Standard statistical methods were used to compare the performance of the Simplexa qPCR to that of the GASD assay. This study was approved by the CLS Microbiology Research Committee.

The annual costs of testing for GASD in our laboratory were compared to both Simplexa qPCR and standard culture. Labor costs were based on the current hourly rates paid by CLS to medical laboratory assistants (MLAs) and medical laboratory technologists (MLTs). Supply costs included the Canadian Goods and Services tax (GST). All reported costs are in Canadian dollars.

RESULTS

A total of 289 individual throat swabs were tested in parallel. Table 1 shows the initial and resolved performance of GASD and Simplexa qPCR. A total of 54 (18.7%) throat samples were GAS positive by both methods. Six additional samples gave discrepant results: 4 were only positive by Simplexa qPCR, and 2 were only GAS positive by GASD. Overall Simplexa qPCR initially had a sensitivity, specificity, efficiency, positive predictive value (PPV), and negative predictive value (NPV) of 96.7%, 100%, 99.3%, 100%, and 99.1%, respectively. After secondary testing of the 6 discrepant samples, Simplexa qPCR compared to GASD had a sensitivity, specificity, efficiency, PPV, and NPV of 100% versus 93.1%, 100% versus 100%, 100% versus 100%, and 100% versus 98.3%, respectively. Threshold cycle (C_T) values for the second PCR on the initially discrepant samples ranged from 31.8 up to 38.1, indicating the presence of a low organism burden.

The component costs of routinely implementing the Simplexa qPCR method are compared to standard culture and GASD in Table 2. Although Simplexa qPCR would be more expensive to use as the primary method in our laboratory jurisdiction, this system would allow all results to be reported the same day and would significantly

TABLE 2 Annual cost of using Simplexa GAS qPCR or the Hologic GAS Direct hybridization assay compared to standard culture[a]

Parameter	Cost of standard culture[b]	Cost of GASD	Difference vs standard culture	Cost of qPCR[c]	Difference vs standard culture	Difference vs GASD
Labor/test	$3.73	$1.56	↓ $2.17 (↓ 58.2%)	$0.97	↓ $2.76 (↓ 74%)	↓ $0.59 (↓ 37.8%)
Materials/test	$0.91	$2.80	↑ $1.89 (↑ 208%)	$8.29	↑ $7.38 (↑ 811%)	↑ $5.49 (↑ 196.1%)
Total cost/test	$4.64	$4.35	↓ $0.29 (↓ 6.3%)	$9.26	↑ $4.62 (↑ 99.6%)	↑ 4.91 (↑ 112.9%)
Monthly costs	$51,545.76	$48,324.15	↓ $3,221.61	$102,869.34	↑ $51,323.58	↑ $54,545.19
Annual costs	$618,549.12	$579,889.80	↓ $38,659.32 (↓ 6.3%)	$1,184,413.94	↑ $615,882.96 (↑ 99.6%)	↑ $654,542.28 (↑ 105.8%)

[a]All costs for each molecular method are calculated in Canadian dollars and based on a total test volume of 11,109 throat samples per month according to CLS workload in 2016. GASD, Hologic GAS Direct hybridization assay; ↑, increase in cost; ↓, decrease in cost.

[b]The total costs per test for standard culture are calculated in Canadian dollars. The overall monthly and annual costs are extrapolated to the total test volume outlined in footnote *a*.

[c]The cost per test is calculated as part of a reagent rental contract that includes the liquid handling system and 3M integrated cycler. Although the cost per test may be lowered by outright purchase of the equipment, there would be significant upfront capital expenditure. Cost per test also does not include the increased cost of using an eSwab as required for qPCR (calculated at $0.69 per unit) compared to the use of a nonflocked liquid Amies swab (calculated at $0.26 per unit).

decrease the labor costs currently being expended for GASD, which is a much more manual procedure. Universal Simplexa qPCR analysis would save an estimated 0.62 full-time equivalent (FTE) of medical laboratory technologist time that could be deployed to other duties. Secondary culture would only have to be performed to provide an antibiotic susceptibility result for GAS-positive patients with a reported history of penicillin allergy. Implementation of Simplexa qPCR would also require capital investment for an automated pipettor (Table 2) in order to efficiently handle sample preparation for >10,345 throat swabs per day, or >125,000 throat swabs per year. Although GASD has a similar cost per test to routine culture in our laboratory setting (Table 2), the routine use of Simplexa qPCR would result in an increased cost per test that is approximately 20% greater than that of either GASD or a culture-based method.

DISCUSSION

Our study is the first to evaluate the use of different types of molecular methods for detection of GAS colonization/infection in patients with suspected pharyngitis. Ours is also one of the first reported evaluations of the Simplexa Direct GAS qPCR assay run in a high-volume (i.e., 96-well) instrument platform. Although culture was not performed in our study, prior studies have documented that GASD has a comparable performance to culture (18, 19). The Simplexa qPCR demonstrated a greater sensitivity than GASD in our study, even though it was always performed after the hybridization assay was complete in order to not compromise the reporting of the routine test method. The two false-negative Simplexa qPCR results are likely due to nondetection of the GAS target due to sample dilution that occurred through the dual processing of the original throat swab and secondary eSwab. Ideally, a separate Amies swab and eSwab would be alternately collected from each patient in order to perform GASD and qPCR, respectively. Our study confirms and enhances the findings of Tabb et al. (22), who prospectively evaluated the Simplexa GAS qPCR against culture in 1,342 individual throat swabs from a patient cohort that had a GAS prevalence of 15.4%. The assay demonstrated 97.4% sensitivity and 95.2% specificity versus culture. Although the positive predictive value was only 72.7%, 46 out of 57 discrepant samples (qPCR positive but culture negative) were confirmed as positive by repeat testing using a bidirectional sequencing method, thus confirming the increased sensitivity of the molecular assay.

Limitations of this study include the relatively small sample size and the fact that both molecular tests were performed from a single throat swab that was preferentially prioritized to initially perform the routine test and not the Simplexa qPCR. Our study design also meant that the Simplexa qPCR was performed in an off-label manner and culture could not be performed. However, the low overall C_T values recorded for the secondary qPCR indicate that the initial "false-negative" Simplexa qPCR result was most likely due to a low remaining organism burden.

Prompt detection of GAS from throat swabs is important not only to initiate antibiotic therapy, but also to prevent the unnecessary use of empirical antibiotics for

viral pharyngitis. Many clinical microbiology laboratories continue to use culture as the "gold standard" method for GAS detection, but this approach is too slow to assist with precise clinical prescription for patients with bacterial pharyngitis. Culture is also labor intensive, and as shown in Table 2 requires far more labor in a high-volume laboratory (i.e., 82.8% more MLA and 41% more MLT hands-on time) than molecular detection. Because of the technical limitations of a culture-based service, many organizations recommend using a two-stage algorithm for GAS testing: a rapid antigen detection test (RADT) is initially used to detect GAS with a secondary culture of initially RADT-negative samples (24). However, using a less-sensitive RADT for initial screening not only introduces the potential for error (25, 26), but the total test cycle may not be truncated, and this approach will be more expensive than culture alone.

Although our laboratory has routinely used molecular GAS detection for more than a decade for rapid same-day screening of a high volume of throat swabs, the annual volume of GASD tests ordered in our region has exceeded the upper limit of tests (i.e., 200 to 250 tests) that can feasibly done within an 8-h shift. Because GASD is a semiautomated procedure that requires processing of throat swabs (i.e., placement in lysis reagent and heating in a 95°C dry water bath for 10 min prior to testing, more MLA time is required to process >10,000 throat swabs/month (i.e., >300 to 350/day). In addition, the GASD procedure requires several manual steps and requires 3 h to complete 96 tests.

Implementation of a commercial real-time PCR method for high-volume GAS detection from throat swabs would address many of these clinical and operational issues. The Focus Diagnostics (now Diasorin, Inc., Italy) Simplexa GAS Direct qPCR assay is currently one of the only real-time PCR tests for high-volume GAS detection that currently has HPB licensing approval in Canada. However, there have recently been several real-time PCR assay for GAS detection approved for smaller sample volumes and/or point-of-care testing licensed in North America, including Solana GAS (Quidel) and the cobas Liat GAS (Roche). The Simplexa qPCR assay system is ideal for a high-volume laboratory setting because it requires no sample preparation, and 95 tests, including the internal control, can be completed in a single instrument run in about an hour.

One drawback of using a molecular method for primary GAS detection in all patients is the loss in ability to subculture GAS isolates in positive cases for routine antibiotic susceptibility testing. GAS-positive samples detected by qPCR would therefore still need to be subcultured in order to perform antibiotic susceptibility testing due to treatment failures or those with penicillin allergy. Culture is also still required in order to detection other beta-hemolytic streptococci (i.e., large-colony group C and group G) or *Arcanobacterium haemolyticum* if requested. Another potential limitation of the routine use of commercial qPCR in Canadian health care jurisdictions that operate on fixed budgets is the substantially increased overall cost per test compared to either a culture-based method or GASD for routine GAS detection in throat swabs. A substantial increase in our laboratory funding from our single payer system is required to fully implement GAS qPCR for routine detection of this organism in the more than 125,000 individual throat swabs received in 2015. Laboratories in the United States that receive reimbursement for testing may not face these financial restrictions. Clinical laboratories that routinely process a high volume (>50,000 per year) of throat swabs for GAS detection should consider implementing Simplexa qPCR for primary GAS diagnosis, depending on the cost limitations of individual health care jurisdictions. Molecular real-time PCR assays with sensitivity and specificity on par with or greater than those of culture have the potential to replace throat swab culture as the gold standard method in clinical laboratories. Rapid point-of-care tests with similar performance could also replace RADTs as standalone tests for near patient testing, thereby improving the clinical care of patients with suspected pharyngitis presenting to emergency departments, urgent care centers, and ambulatory practices.

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T.L. performed all of the diagnostic testing.

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