Highly Sensitive Molecular Assay for Group A Streptococci Over-identifies Carriers and May Impact Outpatient Antimicrobial Stewardship

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Background: Timely, accurate diagnosis of group A streptococci (GAS) pharyngitis prevents acute rheumatic fever and limits antibiotic overuse. The illumigene group A Streptococcus assay (Meridian Bioscience, Cincinnati, OH) is a molecular test for GAS pharyngitis with high sensitivity and specificity. We sought to determine whether the illumigene test is more likely than throat culture to be positive in patients without pharyngeal symptoms and explore the limits of detection of the test.

Methods: Patients 3–17 years of age were eligible if they had no history of pharyngitis or use of antibiotics within the previous 2 weeks; there were no upper respiratory infection symptoms, sore throat or fever and no signs of infection. Culture and illumigene were performed on duplicate throat swabs. Excess lysate from a subset of illumigene tests was evaluated by real-time polymerase chain reaction. Institutional Review Board approval was obtained.

Results: We enrolled 385 patients from February 2016 to October 2017; mean age was 10 yr; 51% were male. Most visits were for health supervision (69%). Significantly more illumigene tests (78/385, 20.3%) than throat cultures (48/385, 12.5%) were positive (χ^2; P =0.0035). Illumigene was "indeterminate" for 3 patients, leaving 382 pairs of swabs for analysis. Results were discordant for 32 of 382 pairs (8.4%); 31 of 32 (97%) were illumigene-positive/culture-negative (McNemar test; $P < 0.000001$). Real-time polymerase chain reaction was negative in 4 of 13 (31%) tested illumigene-positive lysates; the paired culture had been negative in all four. The limit of detection for the illumigene test was 55 colony forming units/mL.

Conclusions: The illumigene test is significantly more likely than throat culture to yield positive results in patients without GAS pharyngitis. Failure to appropriately select patients for testing may negatively impact antimicrobial stewardship efforts without benefit to patients.

Key Words: group A streptococci, pharyngitis, streptococcal carriers, nucleic acid amplification test, antimicrobial stewardship

(*Pediatr Infect Dis J* 2019;38:769–774)

Key Points: The illumigene test is significantly more likely than culture to identify group A streptococci in asymptomatic children and will likely result in overdiagnosis of streptococcal pharyngitis and treatment of carriers. Clinical guidelines are needed to minimize adverse impact on antimicrobial stewardship.

Pharyngeal infection with group A streptococci (GAS) puts patients at risk for acute rheumatic fever (ARF) and other complications. Accurate diagnosis of GAS pharyngitis and prompt antibiotic treatment are the hallmarks of ARF prevention in the US and other developed countries despite very low rates of ARF. The throat swab cultured on a sheep blood agar plate (BAP), pioneered by Breese and Disney in their pediatric office more than 60 years ago,[1] is considered the "gold standard" for diagnosis of GAS pharyngitis in children but takes 18–48 hours to provide a definitive result. Rapid antigen detection tests (RADTs) that take 5–15 minutes to provide a result at the point-of-care (POC) have become first-line tests for GAS since the mid-1980s.[2] RADTs are fairly specific but they have relatively poor sensitivity (83–88%) in a 2016 Cochrane review.[3] Published management guidelines from the American Academy of Pediatrics and the Infectious Diseases Society of America (IDSA) recommend the use of throat culture to confirm negative RADT results in children.[4,5] Following the clinical laboratory improvement amendments of 1988 regulation of physician office laboratories (POLs) most pediatric offices in the US began sending swabs to a central laboratory for confirmatory throat culture rather than processing them on-site.[6,7]

Molecular-based assays for GAS that provide results in <60 minutes have become available for use at the POC. They offer the potential for providing a result much more quickly than needed for a BAP result. These nucleic acid amplification tests (NAATs) are reported to be extremely sensitive and specific, and some are cleared by the U.S. Food and Drug Administration to be used without culture confirmation of negative results.[8] The illumigene group A Streptococcus assay (Meridian Bioscience, Cincinnati, OH) uses loop-mediated isothermal amplification to detect the highly conserved *speB* (streptococcal pyrogenic exotoxin B) gene of GAS on throat swabs. This CLIA-moderate test is cleared by the Food and Drug

Accepted for publication January 21, 2019.

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This work was supported by funds within the Division of Academic General Pediatrics and the Division of Infectious Diseases, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, Illinois.

R.R.T. and S.T.S. received support from Meridian Bioscience Corporation (Cincinnati, OH), the developer of the illumigene test, for a study unrelated to this article. Meridian Bioscience Corporation provided 100 illumigene tests for this study. No one involved in this project received payment to produce it. The other authors have no conflicts of interest to disclose.

R.R.T. designed this study, enrolled patients, supervised all aspects of the study, performed the data analysis and drafted and revised the article. E.J.R. designed this study, enrolled patients and contributed to the article. J.L.R. directed the Special Infectious Diseases Laboratory, performed laboratory studies, managed the data and contributed to the article. R.L.D. enrolled patients, collected throat swabs and reviewed the article. C.L.O. enrolled patients, collected throat swabs and reviewed the article. C.L.L. performed laboratory studies, managed the data and reviewed the article. S.T.S. contributed to study design, data interpretation and article preparation.

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Presented in part at the 2018 Pediatric Academic Societies' Annual Meeting; May 7, 2018; Toronto, Ontario, Canada.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Website (www.pidj.com).

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ISSN: 0891-3668/19/3808-0769
DOI: 10.1097/INF.0000000000002293

Administration as a stand-alone test for GAS pharyngitis; results are considered definitive whether positive or negative. It takes <1 hour to perform using a proprietary countertop device and can be performed in a microbiology laboratory or at the POC in a POL.

In 2014, the microbiology laboratory in our large children's hospital replaced BAP throat culture with the illumigene test for diagnosis of GAS pharyngitis; a back-up illumigene test is used whenever a POC RADT is negative. Subsequent analysis of laboratory records demonstrated that changing to the molecular test was associated with a significant increase in the proportion of tests positive for GAS in the laboratory.[9] The precise reason for the increase was not known, but it seemed likely that the extreme sensitivity of the molecular test was central to our findings.

It is well known that some children harbor GAS in the pharynx persistently without symptoms, sometimes despite having received appropriate antibiotic treatment for previous symptomatic pharyngitis. They are usually considered to be GAS carriers.[10,11] Tests for GAS can be positive when carriers have symptoms of pharyngitis and when they are well. Typically about 10–12% of children with culture-positive pharyngitis may be GAS carriers with an intercurrent nonstreptococcal (usually viral) illness.[12–16] If the illumigene test is more likely than culture to identify GAS in children who are carriers, that would help explain the increase in positive tests we have documented in our laboratory with illumigene. We designed a prospective study to compare illumigene to culture in asymptomatic children.

MATERIALS AND METHODS

Collection and Processing of Throat Swabs

Eligible patients were 3–17 years old visiting our pediatric resident Continuity Clinic for a health supervision visit or follow-up of a noninfectious condition. Subjects were enrolled from February 2016 through October 2017. Patients were excluded if they had current upper respiratory infection symptoms, sore throat, fever or any other signs of infection; history of pharyngitis within the previous 2 weeks and treatment with an antibiotic for any reason within the previous 2 weeks or prior enrollment in the study. With written informed consent (plus assent from patients ≥12 years old) 2 throat swabs were obtained simultaneously by clinic nurses (R.L.D., C.L.O.) or physicians (E.J.R., R.R.T.). The tips were rubbed together or rolled over each other before placing the swabs in transport medium. Each swab pair was placed in a plastic specimen transport bag and kept in the nurses' office at room temperature until delivered by a nurse to our Special Infectious Diseases Laboratory (infectious diseases research laboratory) immediately after each 4-hour clinic session.

Patient age, sex, reason for visit and use of inhaled or systemic corticosteroids were recorded on a numbered case report form (CRF). CRFs were identified by case number only; patient personal identifiers were not collected. Medical records were not reviewed. Results of testing were not used for patient care; were not provided to the patients, parents or clinic physicians and were not recorded in the electronic medical record. Consent/assent forms were not numbered and were kept separate from the CRFs. The enrolled subjects constitute a convenience sample affected by clinic patient volume, investigator availability and family willingness to participate.

In the infectious diseases research laboratory, 1 swab was randomly chosen for performance of BAP culture and 1 for the illumigene test. Culture was performed using 5% sheep blood agar incubated at 35°C in room air for up to 48 hours. GAS identification was based on colony morphology, Gram stain, catalase test and serogrouping using PathoDX Strep Grouping (Remel, Lenexa, KS). GAS growth was semiquantified from 1+ (lightest) to 4+ (heaviest). The illumigene test was performed following manufacturer's instructions. The proprietary illumigene countertop device provides test results in about 50 minutes as positive, negative or indeterminate.

Illumigene Limit of Detection

The illumigene test uses 0.05 mL of the nucleic acid lysate prepared in the extraction step, leaving 0.5–1 mL of lysate. Excess lysate from 13 illumigene-positive swabs was frozen at –70°C for subsequent evaluation using real-time polymerase chain reaction (RT-PCR) (genesig; Primerdesign, Southampton, United Kingdom) targeting the *sclA* gene (streptococcal collagen-like surface protein). We used 0.2 mL of excess illumigene lysate for nucleic acid extraction and 25 uL of the subsequent nucleic acid to perform each RT-PCR. A control was included in each PCR run.

GAS concentration [colony forming units (CFU)/mL] on these 13 throat swabs was calculated as follows. Serial dilutions of GAS control strain ATCC 19615 were prepared in phosphate-buffered saline. Nucleic acid was extracted using 0.2 mL of each dilution. RT-PCR was performed using 25 uL of the subsequent lysate. A standard curve was created using the RT-PCR cycle threshold score at each concentration of GAS. The RT-PCR cycle threshold scores of the 13 illumigene lysates prepared from study throat swabs were compared with the standard curve to calculate the GAS concentration on each swab.

All study procedures, including consent forms and assent forms, were approved by the Lurie Children's Hospital Institutional Review Board. The χ^2 test and McNemar test for paired data were used for data analysis.

RESULTS

We enrolled 385 subjects for 21 months, varying from 0 to 53 subjects per month (no patients were enrolled in 4 months: June and August 2016; January and February 2017). Positive illumigene tests exceeded positive cultures in 11 of 17 months in which subjects were enrolled; in each of the remaining 6 months, there were equal numbers of positive cultures and positive illumigene tests, 0–2 in each (Supplemental Table, http://links.lww.com/INF/D432). Table 1 shows the characteristics of the subjects. The most common primary reason for the clinic visit was health supervision (69%). Patient sex and age did not influence the likelihood that either test would be positive. Thirteen of 385 patients (3.4%)

TABLE 1. Patient Population

Patient Characteristics	Enrolled	Culture-positive	Illumigene-positive
Number	385	48 (12.5%)*	78 (20.3%)*
Age (yr)			
Mean	10	9.6	9.6
Median (range)	10 (3–17)	9 (5–16)	9 (4–17)
Sex (n = 383)†			
Male	195 (51%)	23	39
Female	188 (49%)	25	39
Used corticosteroids (n = 13)			
Inhaled	11	1	1
Oral	1	0	0
Unknown type	1	0	0
Reason for visit (n = 381)†			
Health supervision	264 (69%)	28	46
Miscellaneous follow-up	100 (26%)	18	27
ADHD‡	17 (4.5%)	2	5

*Proportion culture-positive vs. illumigene-positive: χ^2, $P = 0.0035$.
†Missing data: sex (2), reason for visit (4).
‡Attention deficit hyperactivity disorder.

TABLE 2. GAS Test Results for 382 Paired Throat Swabs

GAS Test*	Culture-positive	Culture-negative	Total
Illumigene-positive	47	31	78
Illumigene-negative	1	303	304
Total	48	334	382

Odds ratio = 31 (95% confidence interval = 4.2–227.1)
*McNemar test (2-tailed), $P < 0.000001$.

had used corticosteroids within the previous 2 weeks: 11 had used inhaled steroids; 1 oral steroids and 1 unknown steroid. Twelve of 13 patients (92.3%) who used steroids had a negative culture and a negative illumigene test. One patient who had used an inhaled corticosteroid was positive by both culture and illumigene.

Significantly more illumigene tests (78/385, 20.3%) than standard throat cultures (48/385, 12.5%) were positive in these asymptomatic children (χ^2, $P = 0.0035$). The growth of GAS on 47 of the 48 positive BAPs was semiquantified from 1+ to 4+. Only 11 (23%) of 47 were scored as 1+ growth; 25 (53%) were considered to be 3+ or 4+.

Three swabs yielded an "indeterminate" illumigene test result (all 3 paired swabs were culture-negative), leaving 382 pairs. Table 2 shows the results of culture and illumigene testing of the paired swabs. Results were discordant for 32 of 382 pairs (8.4%), of which 31 of 32 (97%) were illumigene-positive/culture-negative. A positive result was significantly more likely with the swab chosen for the illumigene test than with the one used for culture (McNemar test, $P < 0.000001$; odds ratio, 31; 95% confidence interval, 4.2–227.1). There were no differences in the age or sex of the 47 patients for whom both tests were positive and the 31 who were illumigene-positive/culture-negative (data not shown).

Frozen excess lysate from 13 illumigene-positive swabs was thawed and then evaluated using RT-PCR targeting the *sclA* gene of GAS. Table 3 displays the PCR results alongside the paired culture and illumigene results. Notably, in this small subset of illumigene-positive swabs 4 of 13 lysates (31%) yielded negative PCR results; the paired throat culture had been negative in all four (patients J–M). The calculated GAS concentration for 6 of 9 PCR-positive lysates (55–291 CFU/mL; patients A, D–H) was lower than the limit of detection described in the illumigene package insert (400–430 CFU/mL).[17] The estimated limit of detection (55 CFU/mL) in this small sample was from a culture-negative swab (patient F) and was lower than the GAS concentration associated with the culture plate that had 1+ growth of GAS (291 CFU/mL, patient A). Figure 1 shows the relationship between PCR cycle threshold and calculated GAS concentration (CFU/mL) among PCR-positive lysates (semi-log scale).

DISCUSSION

This prospective study demonstrates that the illumigene test is significantly more likely than culture to identify GAS in patients who do not have clinical evidence of GAS pharyngitis. The 12.5% of swabs that were culture-positive from patients without signs or symptoms of pharyngitis or other infection approximate rates of GAS carriage reported in the literature.[12–16] In contrast, more than 20% of paired swabs were positive using the illumigene test, a 60% increase over culture. GAS test results were not influenced by patient sex, age or use of corticosteroids within the previous 2 weeks.

The subjects enrolled in this study constitute a convenience sample in clinic for a health supervision visit or follow-up of a non-infectious problem. Although monthly enrollment was extremely variable, there was never a month in which there were more positive cultures than positive molecular tests. This study was designed to minimize the potential for bias in patient selection and swab sampling error. Cultures were performed in standard fashion by experienced laboratory personnel and all illumigene tests were performed according to the manufacturer's instructions. We have no data on the prevalence of acute pharyngitis in the community during the nearly 2-year course of this study, and we did not review medical records of the study subjects.

In 3 hospital laboratory-based studies,[18–20] (1 performed at our institution[19]) both sensitivity and specificity of illumigene were >95% when compared with culture (using PCR to adjudicate discordant culture and illumigene results). In contrast, illumigene sensitivity was 87% when used in the New Zealand school-based Rheumatic Fever Prevention Programme, although the manufacturer's

TABLE 3. Results of RT-PCR of Excess Lysate From 13 Positive Illumigene Tests

Patient	Throat Culture*	Illumigene Test†	RT-PCR‡	RT-PCR Cycle Threshold	GAS Concentration (CFU/mL§)
A	Positive 1+	Positive	Positive	32.9	291
B	Positive 3+	Positive	Positive	27.5	9856
C	Positive 4+	Positive	Positive	29.5	2545
D	Negative	Positive	Positive	35.0	73
E	Negative	Positive	Positive	34.3	118
F	Negative	Positive	Positive	35.4	55
G	Negative	Positive	Positive	32.9	278
H	Negative	Positive	Positive	33.9	144
I	Negative	Positive	Positive	32.0	503
J	Negative	Positive	Negative	—	—
K	Negative	Positive	Negative	—	—
L	Negative	Positive	Negative	—	—
M	Negative	Positive	Negative	—	—

*Semiquantified throat culture.
†Illumigene target sequence is *speB*.
‡Real-time PCR (RT-PCR) target sequence is *sclA*.
§GAS concentration (CFU/mL) was calculated by comparing RT-PCR cycle threshold scores of lysates from positive illumigene tests (subject throat swabs) to a standard curve created from RT-PCR cycle threshold scores of known GAS concentrations (see Methods).

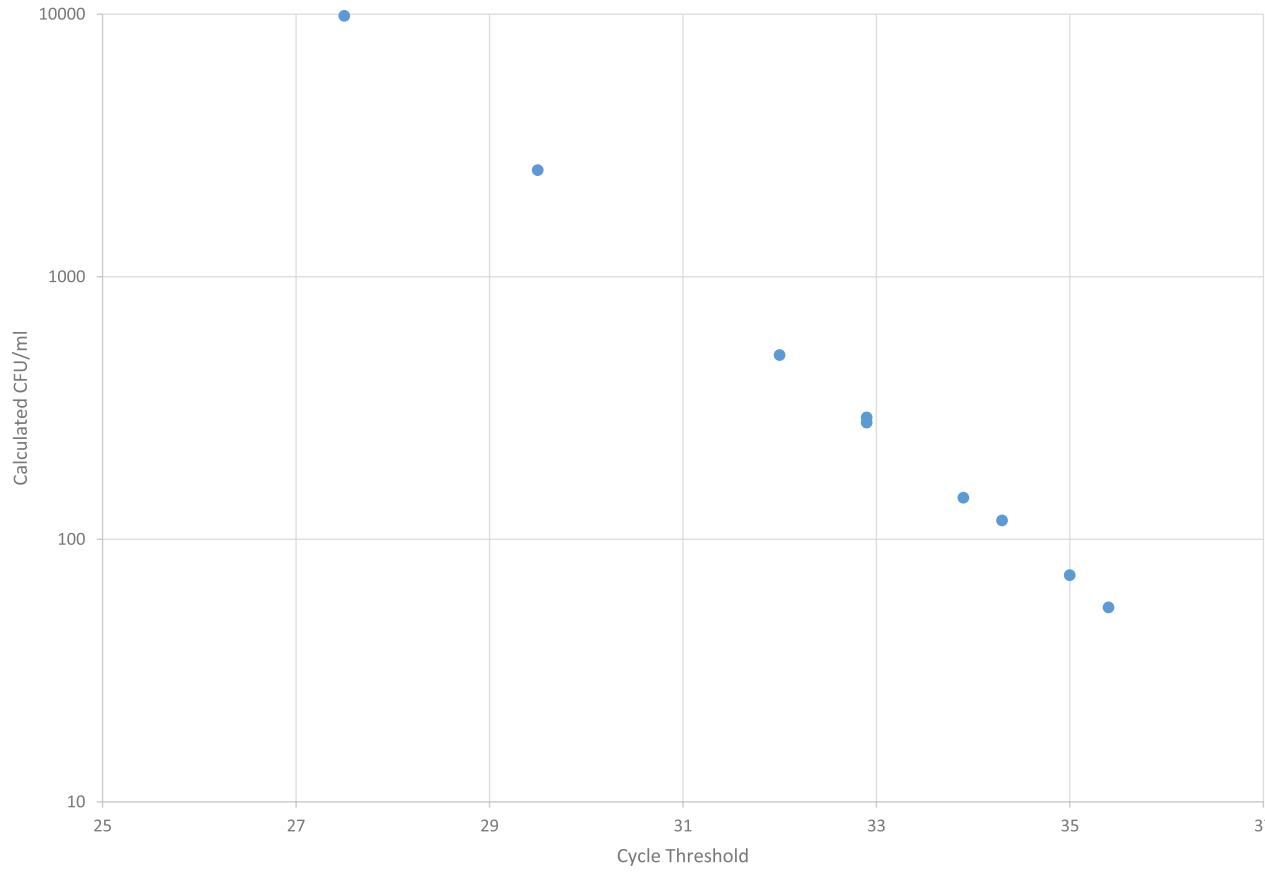

FIGURE 1. Relationship between RT-PCR cycle threshold and calculated GAS concentration (CFU/mL) for 13 illumigene-positive throat swabs (semi-log scale)*.

recommended procedures were not precisely followed in that study.[21] After our hospital microbiology laboratory replaced culture with the illumigene test for back-up, in a retrospective study of RADT-negative emergency department patients we found that the proportion of tests that were positive nearly doubled from 8.4% with culture to 16.5% with illumigene.[9] The results of the present study are consistent with the hypothesis that the sensitivity of the illumigene test is such that it can identify GAS more readily than occurs with standard throat culture, especially in patients with very low streptococcal burden.

A recent report of children enrolled in a longitudinal study of pediatric autoimmune neuropsychiatric disorder associated with streptococci found that some subjects developed an antibody response to GAS after asymptomatic acquisition of pharyngeal GAS.[22] Those findings imply that some asymptomatic GAS-positive children might have "subclinical" infection and are therefore potentially at risk for GAS sequelae.[23] We do not know whether some of the test-positive patients in this study had subclinical GAS infection. While illumigene might be more likely than culture to identify patients with asymptomatic (subclinical) infection, this would not explain the increase in positive tests we previously identified among patients with symptomatic pharyngitis.[9] Moreover, there is no evidence that failure to identify "subclinical" acquisition of pharyngeal GAS has been associated with outbreaks of suppurative or nonsuppurative GAS disease (although some ARF patients are unable to recall sore throat[24]) and there is no realistic way to prospectively identify subclinically infected children.

Individuals who harbor GAS in the pharynx when asymptomatic are usually considered to be colonized rather than infected with GAS.[10,11] The pathophysiology of persistent pharyngeal colonization (carriage) with GAS is unknown. Carriers traditionally have not been considered to be at risk for poststreptococcal sequelae and they have not often been identified as a source for GAS disease outbreaks.[11] Current guidelines do not recommend identification or treatment of carriers except in special circumstances.[4,5] When patients with clinical pharyngitis test positive for GAS it is difficult to distinguish those with acute infection from those who are GAS carriers with intercurrent illnesses. Consequently, carriers with intercurrent viral illness are often treated with antibiotics if a test is positive. Unnecessary swabbing[8] and overuse of antibiotics to treat nonstreptococcal pharyngitis is well-documented.[25] Any test that increases identification of GAS in asymptomatic patients, as occurs with the illumigene test, will increase unnecessary antibiotic treatment of carriers when they have pharyngeal symptoms not caused by GAS, such as when they have viral pharyngitis.

Increased identification of carriers might not be the only reason that the illumigene test is positive more often than culture. In the small subset of 13 illumigene-positive lysates that we tested by PCR, 4 (31%) were negative (and the paired swabs had been culture-negative). Culture-negative/illumigene-positive/PCR-negative results have been noted in other studies.[18–20] The illumigene test has very high specificity but it sometimes yields false positive results for unknown reasons. *SpeB*, the target gene for the illumigene test, has been identified as a highly sensitive and specific PCR target in GAS.[26] It is found universally in *Streptococcus pyogenes* and not in

other streptococci or other pharyngeal bacteria; there is no reason to suspect that false-positive illumigene tests reflect amplification of *speB* from other bacteria.

Both BAP culture and RADT exhibit spectrum effect, that is, test results are affected by disease presentation. Patients with signs and symptoms more consistent with GAS pharyngitis are more likely to have a positive test.[27–29] Test sensitivity is therefore directly related to pretest probability. Clinicians can take advantage of this phenomenon by limiting swabs for RADT and culture to patients with findings more consistent with bona fide GAS pharyngitis,[29] as recommended by the American Academy of Pediatrics and IDSA.[4,5] It is not known if the illumigene test exhibits spectrum effect.

Clinical microbiology increasingly relies on molecular methods for diagnosis of infectious diseases. In 2018, IDSA and the American Society of Microbiology published a comprehensive guide to microbiology testing that includes NAATs for GAS pharyngitis.[30] The American Academy of Microbiology has released a report of a 2016 colloquium on diagnostic testing focused on POC and rapid diagnostic methods, including NAATs.[31] Both documents note that clinical guidelines for molecular testing for GAS pharyngitis are lacking. Nevertheless, GAS molecular tests are available to be used in microbiology laboratories and POLs and an argument has been made that NAATs should supplant BAP culture for evaluation of patients with pharyngitis.[32] Our data indicate that caution is needed before NAATs fully replace culture for GAS.

Tests using methods other than loop-mediated isothermal amplification to amplify various GAS nucleic acid sequences are commercially available and some are CLIA-waived.[8] While published reports of these tests include their sensitivity and specificity,[31–36] we are not aware of any data (other than those we present here) that address the identification of GAS in patients who do not have GAS pharyngitis, either asymptomatic patients or GAS carriers with intercurrent illness. The performance of all GAS molecular tests in routine clinical practice, especially in POLs, will require monitoring[8]; their reported very high sensitivity strongly suggests that they are likely to behave much like the illumigene test. The benefit of delivering "definitive" results to patients more rapidly than possible with culture must be tempered by the reality that highly sensitive GAS molecular tests have the potential to conflict with efforts at antimicrobial stewardship, especially in areas with low rates of ARF.

CONCLUSIONS

Illumigene is significantly more likely than the time-honored BAP culture to identify GAS in healthy children, expanding on our observation that illumigene was significantly more likely than culture to identify GAS in RADT-negative emergency department patients with pharyngitis.[9] As with multiplex respiratory viral panels, identifying the presence of a pathogen by amplifying nucleic acid found in the nonsterile upper respiratory tract may not be equivalent to diagnosing infection.[8,37,38] All of the patients in this study were asymptomatic; those with positive GAS tests might have been subclinically infected but it is likely that most were asymptomatic GAS carriers. Some had false-positive illumigene results. Additional studies of GAS molecular tests and new clinical guidelines are needed to define the role of these tests in clinical care. Until we have better understanding of GAS molecular tests, clinicians in North America and most high resource countries should heed expert advice to avoid testing patients at low risk for ARF (age <3 years) or unlikely to have bona fide GAS pharyngitis because their signs and symptoms are more consistent with viral illnesses (cough, laryngitis, croup, conjunctivitis, rhinorrhea and diarrhea).[4,5,8] Diagnostic testing should focus on patients who are most likely to have pharyngitis caused by GAS, placing them at risk for development of ARF: age ≥3 years with signs and symptoms such as fever, cervical adenitis, pharyngeal/tonsillar exudates and/or rash of scarlet fever. In settings with high rates of ARF, this approach may need adjustment.

ACKNOWLEDGMENTS

The authors thank Ram Yogev, MD provided advice on study design and methodology; Sarah Gioia and Stephanie Pelligra, MPH provided essential administrative assistance.

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