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ORIGINAL ARTICLE

Rapid Molecular Diagnosis of Group A Streptococcus with a Novel Loop Mediated Isothermal Amplification Method

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SUMMARY

Background: **Group A Streptococcus (GAS) is the most common bacterial cause of acute tonsillopharyngitis. In this study, it was aimed to evaluate the performance of a novel Loop Mediated Isothermal Amplification (LAMP) method in the rapid diagnosis of GAS in samples taken from children with a pre-diagnosis of acute bacterial tonsillopharyngitis by comparing it with culture and rapid antigen test (RAT) methods.**
Methods: **A total of 100 throat swab samples taken from children at the pediatrics outpatient clinic with suspected tonsillopharyngitis were included in the study. Throat swab samples were analyzed by RAT, throat culture, and LAMP method. GAS suspected colonies were identified with MALDI-TOF MS system. The isothermal amplification reaction for LAMP was conducted by a novel LAMP instrument.**
Results: **According to the results of throat cultures; 53 of them were positive and 47 were negative in terms of GAS. Six (11.32%) of the culture positive samples were found to be negative by the RAT (sensitivity; 88.68%, specificity 100%). While the antigen test was positive, no culture negative sample was detected. One of the culture positive samples was found negative by LAMP. In two samples, while throat culture was negative, it was observed that LAMP was positive (sensitivity; 98.11%, specificity; 95.74%). In one of these samples, the bacteria grown in the culture were identified as *Streptococcus dysgalactiae* by mass spectrophotometry.**
Conclusions: **In this study, it was determined that the LAMP method used in the diagnosis of throat infections caused by GAS has high sensitivity and specificity. We believe that the instrument is easy to use, low cost, portable, and adaptable to point of care tests. There are very few studies in the literature regarding the use of the instrument in this field, and it should be evaluated in terms of its usability in daily practice with new studies.**
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KEY WORDS

Group A Streptococcus, *Streptococcus pyogenes*, bacterial tonsillopharyngitis, throat culture, rapid antigen test

INTRODUCTION

Acute tonsillopharyngitis is one of the most important reasons for admission to primary health care institutions and emergency services, especially in the pediatric population. Group A beta hemolytic Streptococcus *(Strep-*

tococcus pyogenes) is the most common bacterial cause of acute tonsillopharyngitis. In a cohort study conducted in Australia, the incidence of pharyngitis caused by Group A Streptococcus (GAS) in children aged 5 - 12 years was reported to be 13 per 100 people per year [1]. Group A Streptococcus pharyngitis is an important public health problem. In addition to acute symptoms such as sore throat and fever, GAS may cause suppurative complications such as peritonsillar abscess and non-suppurative complications such as rheumatic fever [2].

There are some difficulties in the diagnosis of pharyngitis caused by GAS. Signs and symptoms of GAS pharyngitis are often indistinguishable from viral agents and other causes of sore throat. Symptoms or findings alone are not sufficient but only helpful for the correct diagnosis of GAS pharyngitis [3]. From this point on, Centor criteria including four clinical features including tonsillar exudate, swollen sensitive anterior cervical lymph nodes, fever, and absence of cough are used in the diagnosis [4]. However, the positive predictive value of cases meeting 3 or 4 of the Centor criteria is around 50%, and therefore, treatments planned according to these criteria lead to overuse of antibiotics [5]. Therefore, it is recommended to see the laboratory result of the presence of GAS in order to avoid unnecessary use of antibiotics in the treatment of GAS pharyngitis. The gold standard method in the laboratory diagnosis of GAS pharyngitis is throat culture [6]. However, the disadvantages of this test are that it takes a long time, up to 24 - 48 hours for the result of the test, and the need for the second visit of the patient [7].

In recent years, the use of the Loop Mediated Isothermal Amplification (LAMP) method in the laboratory diagnosis of infectious diseases has been increasing [8,9]. With the LAMP method, even a very small copy number of the target gene sequence can be made up to 109 copies in just one hour [10]. Different from traditional PCR, LAMP significantly increases the specificity of amplification by using 4 - 6 independent primers prepared to recognize 6 - 8 different regions of the target DNA. The LAMP method has advantages such as not needing a thermal cycler, giving results in a short time, not requiring additional devices, a simple laboratory infrastructure being sufficient, and the simplicity of analysis due to the addition of some indicators sensitive to pH change to the reaction, so it has the most widespread use potential for the future, especially for point of care (POC) tests [11].

In this study, it was aimed to evaluate the performance of a new LAMP method in the rapid diagnosis of GAS in samples taken from children with a pre-diagnosis of acute bacterial tonsillopharyngitis by comparing it with culture and rapid antigen test methods.

MATERIALS AND METHODS

Ethical approval was obtained from the ethics committee of Sakarya University Faculty of Medicine for this study (date: 09.10.2019, no. 16214662/050.01.04/141). A total of 100 samples taken from children admitted to the Sakarya University Training and Research Hospital Pediatrics outpatient clinic between April and December 2019 with suspected tonsillopharyngitis were included in the study. Here, outpatients between the ages of 0 - 18 are evaluated by a pediatrician. Like all other departments of our hospital, this department is located in the city center and serves the entire city population of approximately 1 million. The sample group was selected from patients with symptoms such as fever, sore throat, difficulty in swallowing, and findings such as redness (or crypts) in the tonsils.

Three swab samples (first two with cotton culture swab, third with dacron swab) taken from the tonsils and, if any, crypts of the patients were sent to the microbiology laboratory within 1 hour. From the first of the swabs, Strep A rapid antigen test (BioStar, Boulder, Colorado) was studied. A throat culture was performed from the second swab sample. For this, 5% sheep blood agar was inoculated and the plates were incubated at 35 ± 2°C and evaluated at the end of 16 - 18 hours. GAS suspected colonies were identified (pure culture was obtained by taking passages when necessary) with MALDI-TOF MS system (VITEK MS, Biomerieux, France). The third swab was kept at +4°C (maximum 3 days) to be used in molecular tests. DNA isolation from swabs was performed with the Qiamp DNA mini kit (Qiagen, Germany) in accordance with the manufacturer's instructions and the DNA samples obtained were stored at -80°C until molecular tests were studied.

The isothermal amplification reaction for LAMP was conducted by a novel LAMP instrument (TUBITAK Biodmes, Turkey) (Figure 1), and colorimetric isothermal master mix were used. The instrument is suitable for POC use due to its convenient dimensions (approximately 25 cm in length, 13 cm in width, 400 grams in weight) and includes 6 wells sized to accommodate 0.2 milliliter PCR tubes. Six samples (4 patient samples, 1 positive, 1 negative control) can be run at the same time.

Primers targeting the specific region of *S. pyogenes* were designed with the PrimerExplorer V5 program (Eiken Chemical Co., Tokyo, Japan). Since the study is at the patent stage, the primer designs could not be disclosed. A 203 bp region in the spy 1,258 gene was chosen as the target. For LAMP study, 12.5 µL WarmStart® LAMP 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 2.5 µL primer mix, 2 µL template DNA, and 8 µL pure water were placed in the wells of the instrument with 0.2 milliliter PCR tubes with a total volume of 25 µL. The reaction was carried out in LAMP instrument at 65°C for 45 minutes. Four patient samples, one negative, and one positive control were studied in each study. At the end of the study, reaction

Table 1. Comparison of results according to Throat Culture, Rapid Antigen Test, and LAMP methods.

		Throat culture *		
LAMP		Positive	Negative	Total
	positive	52	2	54
	negative	1	45	46
GAS antigen test	positive	47	0	47
	negative	6	47	53
	total	53	47	100

*** - Reference method, LAMP - Loop Mediated Isothermal Amplification.**

Table 2. Diagnostic accuracy of LAMP and rapid antigen test according to standard method as a gold standard.

Assays	AUC	Sensitivity	Specificity	PPV	NPV
LAMP	0.943	98.11	95.74	96.30	97.83
GAS antigen test	0.963	88.68	100.00	100.00	88.68

AUC - Area under the ROC curve, PPV - positive predictive value, NPV - negative predictive value, LAMP - Loop Mediated Isothermal Amplification, GAS - Group A Streptococcus.

Figure 1. The recently developed novel LAMP Instrument (TUBITAK Biodmes, Turkey). (This device was developed with the support of TUBITAK project fund).
https://twitter.com/tubitak/status/1372138914388008970)

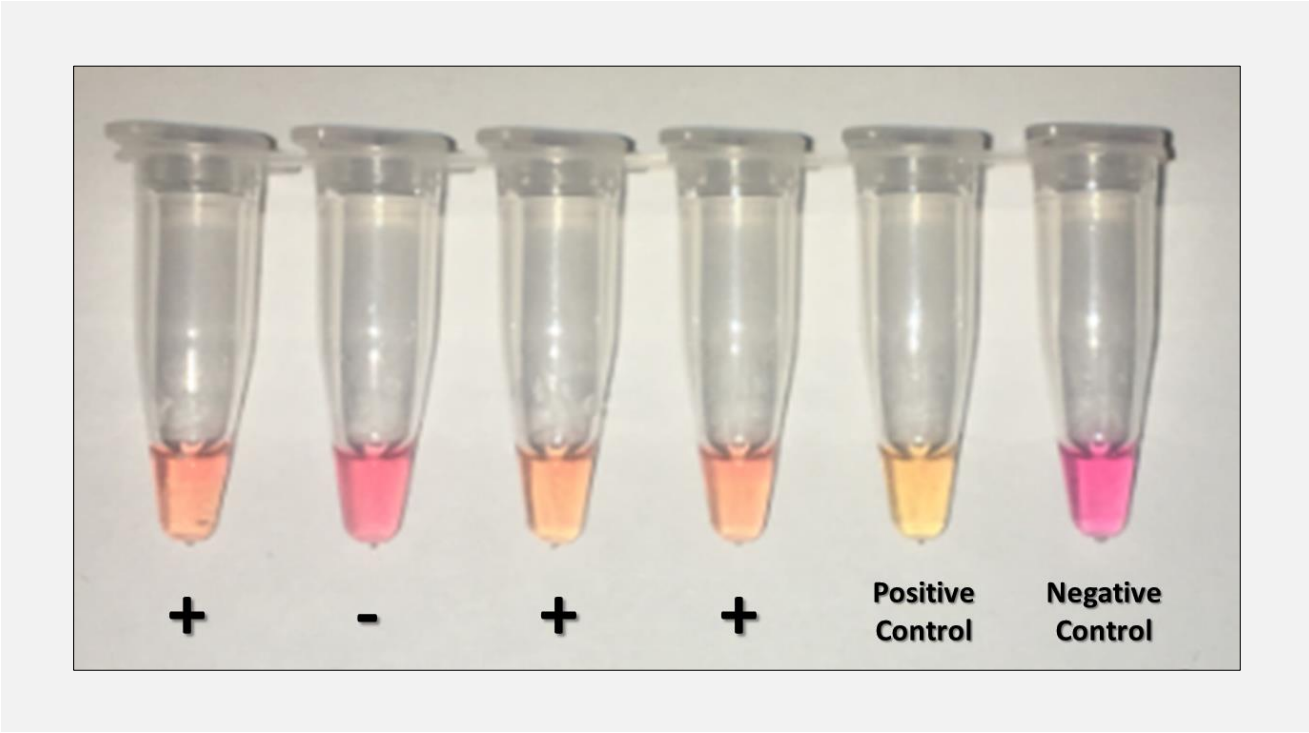

Figure 2. Evaluation of the samples at the end of the LAMP reaction (positive, negative, positive, positive, positive control, and negative control, respectively).

tubes were lined up on a white background and analyzed by naked eye. There is an indicator in the reaction mixture that changes color according to pH changes (phenol red). The Colorimetric Master Mix contains a low-Tris reaction buffer with all necessary cofactors for LAMP including *Bacillus subtilis* DNA Polymerase and Phenol Red for pH detection of LAMP. This system is designed to provide a fast, clear visual detection of amplification based on the production of protons and subsequent drop in pH that occurs from the extensive DNA polymerase activity in a LAMP reaction. Therefore, positive samples were observed as yellow-orange and negative samples as pink/light pink (Figure 2).

In our study, we also compared LAMP performance with Real Time PCR in 50 samples. Real Time quantitative PCR was carried out in 20 µL, containing 12 µL Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA), 1 µL forward primer, 1 µL reverse primer, 6 µL nuclease free water, and 2 µL templete DNA. PCR reaction was performed with a Rotor Gene 5plex HRM (Qiagen, Germany) with the following conditions: 94°C for 10 minutes, and 45 cycles of 94°C 30 seconds and 60°C 1 minute.

Statistical analysis

The area under the ROC curve (AUC), sensitivity, specificity, and positive and negative predictive values were calculated for predictive performance of LAMP and rapid antigen test according to standard method as a gold standard. A p-value < 0.05 was considered significant. Analyses were performed using commercial software (MedCalc Statistical Software version 15.6.1, Med Calc Software bvba, Ostend, Belgium).

RESULTS

Of the patients, 58 were male and 42 were female, and all of them were between the ages of 4 and 18 (mean age; 10.2 years). According to the results of throat cultures of 100 patients with suspected GAS tonsillopharyngitis, 53 of them were positive and 47 were negative in terms of GAS (Table 1).

Six (11.32%) of the culture positive samples were found to be negative by the RAT. While the antigen test was positive, no culture negative sample was detected. When culture is accepted as the gold standard, the sensitivity of the RAT was 88.68% and the specificity was 100% (Table 2).

One of the culture positive samples was found negative by LAMP. In two samples, while throat culture was negative, it was observed that LAMP was positive. In one of these samples, the bacteria grown in the culture were identified as *Streptococcus dysgalactiae* by mass spectrophotometry. It was found that in 97 (97.0%) of the samples, LAMP results were consistent with the results of throat culture, which is the gold standard method. When culture is accepted as the gold standard, the

sensitivity of LAMP was 98.11% and specificity was 95.74%.
The positive results of 50 samples that were studied in quantitative real time PCR are 100% compatible with LAMP. It has been observed that LAMP can successfully detect samples as low as 10^2 cfu/mL. It has been observed that LAMP can successfully detect even samples with bacteria as low as 10^2 cfu/mL.

DISCUSSION

GAS is an important human pathogen worldwide [12, 13]. In addition to throat and skin infections, it may cause complications such as streptococcal toxic shock syndrome, scarlet fever, and necrotizing fasciitis [14, 15]. In addition, the correct diagnosis of throat infections caused by GAS is important in terms of reducing unnecessary antibiotic use and possible antibiotic resistance development [16].
Culture-based classical diagnostic techniques, which are the reference methods, are time consuming. False-negative results can be obtained in GAS rapid antigen detection and other serological methods [17,18]. Therefore, faster, more sensitive and accurate methods are needed for diagnosis. Molecular diagnostic methods are valuable because they have high sensitivity and specificity rates. However, molecular methods such as PCR and real-time PCR used today cannot be widely used in all laboratories because they are expensive and require some extra instrumentation [19].
In the LAMP method that amplifies DNA under isothermal conditions, there are at least four specially designed primers. These primers are linked by recognizing six different regions of the target, and the target gene region is replicated by automatic cycles thanks to the strand displacement mechanism of DNA synthesis using *Bacillus subtilis* DNA polymerase. LAMP reduces the need for complex equipment compared to many amplification methods including PCR. It also has low operating costs and short turn-around times. Having the potential to revolutionize molecular biology, LAMP stands out as a low-cost genetic analysis tool, especially for low/middle-income countries [11]. In addition, LAMP is an easily adaptable method for POC tests, as it does not require a thermal cycler. In this way, it is possible to study the test in areas with limited access to extensive/ large microbiology laboratories and even in polyclinic rooms.
In this study, the performance of the LAMP method in the rapid molecular diagnosis of throat infections caused by GAS was investigated using a recently developed modern LAMP instrument (Biodmes, Turkey). This instrument has a touch screen and from this screen, temperatures, time, and color changes at the end of the reaction can be followed. This instrument, which can provide results in a total of 45 minutes, enables the reaction to take place at 65℃ by acting as a heat block, and also detects the color change due to the pH change in the colorimetric master mix and reflects this result to the screen on the instrument. In this way, the reaction can be followed simultaneously without the need for a separate analysis process, and if the color change is observed, the reaction can be terminated when desired [8-10]. It is a small sized instrument and suitable for POC use.
The Rapid Antigen Test, which was developed to shorten the turn around time of throat culture, has been in clinical use for about 30 years. With a positive RAT result, antibiotic treatment against GAS can be started without waiting for the culture result. However, the most important disadvantage of using these tests is their low sensitivity. In the studies conducted, the sensitivity and specificity of RATs used in the diagnosis of GAS tonsillopharyngitis varies between 65.6 - 96.4% and 68.7 - 99.3%, respectively [20].
There are many studies in the literature about the rapid diagnosis of GAS. Lean et al. examined 23,934 patient results in a meta-analysis they conducted on rapid diagnosis methods of GAS and reported the sensitivity and specificity results varying according to the principle used by the rapid test. Sensitivity has been reported as 59 - 96% for serology-based rapid tests, and 89 - 96% for molecular-based rapid tests. The specificity rates were reported as 94% for serological diagnostic methods and 99% for molecular methods [21]. In our study, the sensitivity of the LAMP and RAT was 98.11% and 88.68%, and the specificity was 95.74% and 100%, respectively. In the identification of one of the 2 false positive samples with LAMP, *S. dysgalactiae* has grown in the culture. However, this bacterium has high genome homology with *S. pyogenes* and is among the C/G group Streptococcus, another important bacterial causative family of acute tonsillopharyngitis [22]. Considering the sensitivity results, it can be said that LAMP is suitable for use in laboratories where the culture-based method, which is the reference method, cannot be performed.
In a study by Altındis et al., 36 GAS strains, throat swabs of 8 patients with GAS detected in simultaneous throat culture, and one standard strain (*S. pyogenes* ATCC 19,615) were studied by LAMP method. The researchers reported that all procedures were completed within 70 minutes with LAMP, 97.2% of the GAS strains (n = 36) isolated from patient samples were found to be positive, and the standard strain sample studied in duplicate was also positive [23].
Jayaratne and Rutherford compared the cultures with LAMP in terms of detecting GAS from throat swabs. They reported the sensitivity of LAMP as 94.1%, specificity as 94.1%, PPV as 80% and NPV as 98.1%. They stated that they obtained LAMP results in 75 minutes and culture results in 24 hours. They also calculated the cost for LAMP and reported that it was 3$ (Canadian dollars) per test [24]. Considering that bacterial pharyngitis is mostly caused by GAS, LAMP stands out as a low-cost molecular analysis method suitable for use especially in low/middle-income countries in the diagnosis of bacterial pharyngitis [25]. It is an important ad-

vantage that it is more affordable than other molecular methods.

The LAMP method has advantages such as having high sensitivity and specificity, being easy to work with, obtaining results in a short time (45 - 70 minutes), and being very small and portable (approximately 25 cm in length, 13 cm in width, 400 grams in weight, and a battery-powered instrument), being applicable to POC that can be performed outside the laboratory. In addition, the ability to analyze and interpret the results on the screen of the instrument without the need for a separate computer and to determine the reaction result by looking at the color change can be considered as its advantages.

Although a commercial kit was used to obtain template DNA in our study, in the literature, it is possible for the sample to participate in the LAMP reaction only by subjecting it to chemical/physical lysis without pretreatment or purification [26]. By simplifying the extraction step, the LAMP method can be used as a highly sensitive molecular rapid test in the diagnosis of not only GAS but also many other infectious agents, after only two pipetting processes that allow adding the sample and primers to the pH-sensitive master mix. For this reason, there is a need for comprehensive studies in which non-extraction methods are tested with many infectious agents, including GAS.

The limitation of our study is that we performed DNA extractions from the samples with a commercial kit rather than a more suitable method for POC studies. In the continuation of our work, we plan to modify the protocol with a simple extraction - or no extraction at all. Another limitation of our study was that quantitative real-time PCR could not be performed in all patient samples due to financial constraints, and this prevented method comparisons between LAMP and real-time PCR and more comprehensive analyses about the detection limit.

As a result, in this study, it was determined that the LAMP method used in the diagnosis of throat infections caused by GAS has high sensitivity, specificity, PPV, and NPV. We believe that the instrument is easy to use, low cost, portable, and adaptable to point of care tests. There are very few studies in the literature regarding the use of the instrument in this field, and it should be evaluated in terms of its usability in daily practice with new studies.

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Declaration of Interest:

None.

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