New Approach to Microscopy of Gram-Stained Urethral Smear: The Kissing Slide Method

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Background: The effectiveness of microscopy of Gram-stained smear (GSS) for the detection of male urethral infection is debatable, especially in cases with low inflammation and no visible urethral discharge. This clinical study compared GSS samples collected with the conventional swab method and our new technique, the kissing slide method, together with polymerase chain reaction results to demonstrate the effectiveness of this new method in men with acute urethritis.

Methods: The study included 64 men who presented to the urology outpatient clinic with complaints of acute urethritis between October 2019 and January 2020. Two GSS samples were collected from each patient, first using the kissing slide method (applying the slide directly to the urethral mucosa), followed by the conventional method. The results were compared with polymerase chain reaction findings.

Results: The patients' mean age was 37.4 ± 7.8 years, and 68.7% had no visible urethral discharge on physical examination. At a GSS threshold of ≥5 polymorphonuclear leukocytes/high-power field, sensitivity values were 60% (95% confidence interval [CI], 42.32%–75.41%) for the kissing slide method and 23.33% (95% CI, 11.79%–40.93%) for the conventional method. At a threshold of ≥2 polymorphonuclear leukocytes/high-power field, sensitivity values with the kissing slide and conventional methods were 80% (95% CI, 62.69%–90.5%) and 50% (95% CI, 33.15%–66.85%) in all patients, and 66.67% (95% CI, 41.71%–84.82%) and 20% (95% CI, 7.047%–45.19%) in cases without visible urethral discharge, respectively.

Conclusion: The new kissing slide method is a noninvasive alternative method that may have better sensitivity than the conventional GSS sampling method in the diagnosis of male acute urethritis. Randomized studies are needed to verify these findings.

Urethritis is an inflammation of the urethra and is often caused by sexually transmitted pathogens.[1] At present, urethritis imposes a substantial global socioeconomic burden because of its transmissible nature. Therefore, diagnosis of acute urethritis is important because undiagnosed patients not only spread disease but also increase the global burden of antibiotic use. Urethritis in men is traditionally diagnosed upon detection of an average of ≥5 polymorphonuclear leukocytes (PMNLs) in 5 high-power fields (HPFs) on microscopic examination of Gram-stained smear (GSS) of a urethral discharge sample.[2] Gram-stained smear is an inexpensive, quick, and easily applied method that not only establishes urethritis diagnosis but also allows for the differentiation of gonococcal urethritis (GU) and nongonococcal urethritis (NGU) based on presence or absence of gram-negative diplococci.[3] However, the effectiveness of GSS has long been a subject of debate. The most common symptoms of urethritis in men are urethral discharge, burning, and itching. Urethral discharge is the main sign of urethral inflammation.[4] Although GSS is sensitive enough in cases of pyogenic urethritis such as GU, its sensitivity is much lower in cases without visible urethral discharge or with low inflammation associated with NGU pathogens.[5,6] For this reason, in its 2015 sexually transmitted disease treatment guidelines, the US Centers for Disease Control and Prevention reduced the positivity threshold value to be used in GSS from ≥5 to ≥2 PMNL/HPF to prevent the underdiagnosis of these cases.[7] Nevertheless, it can be seen from the literature that an alternative method to conventional GSS is still being sought for the diagnosis of urethritis. The ideal method should be inexpensive, be easy to implement, and have high sensitivity in the diagnosis of male urethritis. The aim of this clinical study was to compare the results obtained with GSS samples collected from acute urethritis patients using the conventional method and our new technique, the kissing slide (KS) method, together with polymerase chain reaction (PCR) results to demonstrate the effectiveness of this new method.

MATERIALS AND METHODS

The study included men who presented to the urology outpatient clinic of the Medical Park Antalya Hospital Complex between October 1, 2019, and January 31, 2020, with complaints of burning while urinating, itching, and/or urethral discharge that had started after sexual intercourse. Patients who had received antibiotic therapy because of any infection within the past 4 weeks were excluded from the study. The presence or absence of visible urethral discharge was noted during physical examination. From each of the 64 consecutive patients included in the study, 2 samples were collected for GSS. The penis was not "milked" or otherwise manipulated to express discharge before GSS sampling. Specimens were first obtained with the KS method, then with the conventional method. We ensured that the patients had not urinated within 2 hours before GSS sampling. Immediately after GSS sampling, we first collected urethral samples using a cotton-tipped swab, then first-void urine samples (15 mL) for real-time PCR analysis. All samples were stored at −80°C before analysis.

KS Method

If the patient is uncircumcised, the prepuce is first retracted. With the external urethral meatus exposed, the penis is held in one hand, slightly pulling the glans back from the sides with the first and second fingers to open the external urethral meatus. When the urethral mucosa is clearly visible, the sample is obtained by pressing the slide directly onto the urethral mucosa with the other hand (Fig. 1). We called this method "kissing slide" because of the direct contact between the external urethral mucosa and the slide,

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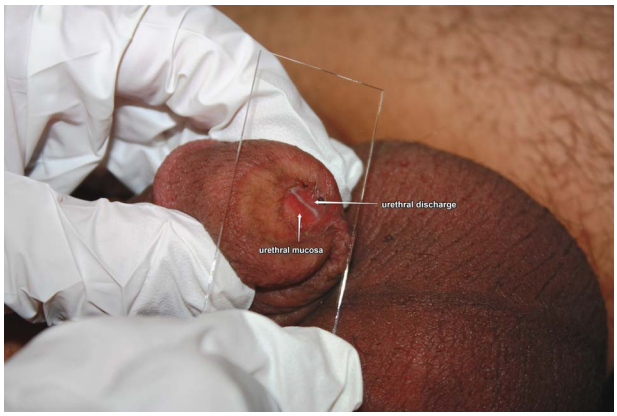

Figure 1. Urethral discharge sample collection using the KS method.

which resembles the act of kissing. In cases where the urethral discharge formed a thick layer on the slide due to its viscosity, it was spread into a thin layer on the slide by moving a thin (1 μL disposable) loop in circular movements in order to facilitate microscopic examination.

Conventional GSS

In the conventional GSS method, a urethral swab sample was obtained by inserting a narrow cotton-tipped swab at least 1 cm into the external urethral meatus and swirling it around 2 to 3 times. The collected sample was spread on a slide.

Slides collected/prepared by both methods underwent standard Gram staining performed by experienced laboratory personnel. All GSS samples were examined by a single experienced microscopist who was blinded to the sample collection method. Polymorphonuclear leukocyte counts were obtained from at least 20 fields at ×100 magnification. The results were evaluated using positivity thresholds of ≥5 and ≥2 PMNL/HPF. The GSS samples of patients without visible urethral discharge were assessed based on a positive threshold value of ≥2 PMNL/HPF. All GSS results were compared with PCR results. In addition, for patients with no visible discharge, GSS results obtained with both sample collection methods were evaluated in terms of agreement with PCR results.

Molecular Analysis

For PCR analysis, DNA was first extracted from the urethral swab and first-void urine samples using the PREP-NA PLUS and PREP-GS PLUS extraction kits (DNA Technology, Moscow, Russia) as per the manufacturer's instructions. The DNA samples were analyzed using a DT Prime 5 Real-Time PCR device, which is manufactured and programmed by the same company.[3] Samples were considered positive upon detection of true pathogens (*Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Trichomonas vaginalis*, and herpes simplex virus type 1 or 2). Samples containing opportunistic pathogens (*Ureaplasma urealyticum*, *Candida albicans*, *Mycoplasma hominis*, *Gardnerella vaginalis*) were evaluated quantitatively in terms of microbial load. A microbial load of $>10^4$ was considered positive, as recommended by the manufacturer.

Statistical Analysis

The OpenEpi version 3.01 (Atlanta, GA) statistics program was used for all statistical analyses. The sensitivity, specificity, and diagnostic accuracy of GSS with the KS and conventional swab methods were calculated within a 95% confidence interval (CI) for each threshold value. For patients with no visible discharge, McNemar test was used to evaluate the agreement between GSS results with both sample collection methods and the results of PCR. $P < 0.05$ was considered significant.

Ethics

The study protocol was approved by the Medical Park Hospital Ethics Committee (approval number: 2019-007). Informed consent forms were signed by all participants before the study. All study procedures were carried out in accordance with the Declaration of Helsinki.

RESULTS

The mean age of the 64 patients included in the study was 37.4 ± 7.8 years. Forty-four (68.7%) of the patients had no visible urethral discharge on physical examination. Using a GSS positivity threshold of ≥5 PMNL/HPF for the diagnosis of acute urethritis, the KS method had sensitivity of 60.00% (95% CI, 42.32%–75.41%) and specificity of 82.35% (95% CI, 66.49%–91.65%), whereas the conventional sampling method had sensitivity of 23.33% (95% CI, 11.79%–40.93%) and specificity of 88.24% (95% CI, 73.38%–95.33%). At a threshold of ≥2 PMNL/HPF, sensitivity and specificity values of the KS method were 80% (95% CI, 62.69%–90.5%) and 79.41% (95% CI, 63.2%–89.65%), whereas those of the conventional method were 50% (95% CI, 33.15%–66.85%) and 82.35% (95% CI, 66.49%–91.65%), respectively. In the 44 patients with no visible urethral discharge, at a GSS threshold of ≥2 PMNL/HPF, the KS method showed sensitivity of 66.67% (95% CI, 41.71%–84.82%) and specificity of 86.21% (95% CI, 69.44%–94.5%), whereas the sensitivity and specificity of the conventional method were 20% (95% CI, 7.047%–45.19%) and 89.66% (95% CI, 73.61%–96.42%), respectively. In addition, in the patients without visible urethral discharge, results obtained with the KS method were consistent with the PCR results ($P = 1.00$), whereas those from conventional swab sampling were not ($P = 0.035$).

The distribution of urethritis pathogens detected by PCR was *C. trachomatis* in 33.3%, *M. genitalium* in 21.2%, *N. gonorrhoeae* in 15.1%, *G. vaginalis* in 12.1%, *U. urealyticum* in 12.1%, *M. hominis* in 3.1%, and *T. vaginalis* in 3.1% of the patients. *C. albicans* and herpes simplex virus type 1 or 2 were not detected in any patient.

DISCUSSION

Acute urethritis is the most common sexually transmitted disease in men.[4] A review of the literature shows that the widespread use of nucleic acid amplification tests like PCR has resulted in significant improvements in the diagnosis and treatment of acute urethritis in recent years.[8] Polymerase chain reaction analysis has enabled the identification of urethritis pathogens that are difficult to identify with conventional methods, using a single sample and with high sensitivity and specificity.[9,10] This development, in turn, has led to more open questioning of the effectiveness of GSS. In fact, the literature includes publications questioning the reliability of the test going back approximately 4 decades.[11] Using a GSS positivity threshold of ≥5 PMNL/HPF, Orellana et al.[6] reported only 26% sensitivity and stated that the absence of leukocytes in GSS cannot rule out a diagnosis of urethritis. In our study, the sensitivity of conventional GSS method at a threshold of ≥5 PMNL/HPF was low (23.3%) and similar to the result obtained by Orellana et al. In a study of Rietmeijer and Mettenbrink[12] found the rate of *C. trachomatis* positivity to be 6.6% at a threshold of 1 PMNL/HPF and significantly higher at 16.2% at 2 PMNL/HPF. Therefore, they suggested lowering the GSS threshold value to 2

PMNL/HPF for the diagnosis of urethritis. In a study by Sarier et al.,[3] lowering the GSS positivity threshold from ≥5 to ≥2 PMNL/HPF resulted in a significant increase in the sensitivity of GSS in diagnosing NGU, whereas no significant change was observed regarding the diagnosis of GU.

It can be seen in the literature that investigators are also seeking alternatives to GSS. Pond et al.[13] reported that flow cytometry of first-void urine was more successful than GSS at diagnosing asymptomatic urethritis. However, the benefit of using flow cytometry is arguable in terms of the benefit-cost ratio because it is both more costly and difficult to perform than GSS. Taylor et al.[14] used methylene blue/gentian violet smears as an alternative to Gram staining for patients with acute urethritis and stated that their results showed 100% agreement with GSS. In a recent study, Jordan et al.[15] compared the GSS results of meatal swabs obtained from one group and urethral swabs obtained from another group of urethritis patients and found no significant difference between the results. However, when patients are separated into groups as in the aforementioned study, comparisons of the results are limited by the difficulty of standardizing the variables between groups. For this reason, the present study was designed to compare the results of the 2 different methods in the same patient group instead of between separate groups.

Gram-stained smear is an established diagnostic method for cases of GU, which often present as pyogenic urethritis. The latest European Association urology guidelines recommend GSS only for the diagnosis of GU.[16] Although there are geographic variations, *N. gonorrhoeae* accounts for only 10% to 20% of urethritis pathogens.[17,18] Therefore, NGU cases are the real problem. Nongonococcal pathogens predominate in urethritis, and unlike in GU, these pathogens can cause different clinical presentations depending on their specific characteristics.[19] Horner et al.[20] reported that at a GSS threshold of ≥5 PMNL/HPF, multivariate analysis showed that symptomatic urethral discharge was observed in only 42% of NGU cases. The absence of urethral discharge will not rule out infection. In this case, the biggest danger is misdiagnosing these patients.

Conventional GSS is swab based, with different types of swabs used to obtain urethral smears. Because there is no standard approach, Dacron swabs, rayon-tipped swabs, plastic loops, blunt metal spatulas, and cotton swab are all used.[4] The use of different materials will undoubtedly affect the sensitivity of the test. In this study, we used cotton-tipped swabs. In our opinion, the cotton swab is a disadvantage from the start in patients with little urethral discharge due to low inflammation. No matter how thin the cotton covering is, some of the discharge is absorbed by this cotton during sample collection. This reduces the yield when spreading the sample on the slide. Unlike conventional swab sampling for GSS, the KS method is based on direct contact and thus uses no intermediate tool to transfer the specimen. This provides a higher yield when transferring discharge to the slide in patients with low inflammation. The most important point to be considered during sample collection using the KS method is to achieve maximal opening of the external urethral meatus by pulling on both sides and applying the slide directly to the urethral mucosa after it becomes visible. The aim is to obtain the maximum yield from the discharge. Conventional urethral swab sampling for GSS involves inserting a swab into the urethra and turning or swirling it around to collect the sample. No matter how gently this is done, it is still an invasive procedure and therefore painful. Especially in patients with no visible discharge, prolonged swabbing in the urethra to obtain a sufficient amount of sample makes the procedure even more painful. For this reason, many patients want to avoid this test both because of the pain during the procedure itself and the burning sensation that occurs when urinating for some period afterward. The KS method is noninvasive because it is not based on swabbing. This makes the procedure painless, which is an important advantage for the patient. In addition, the KS method can also be performed by the patient himself.

Despite advances in technology, GSS remains an important point-of-care test because it is easy to perform and provides rapid results. Although PCR is an effective method for detecting urethritis pathogens, its cost, lack of availability in some centers, and waiting time for results limit its use as a primary test for the diagnosis of acute urethritis. Therefore, we believe that clinicians, patients, and the population at large will benefit from the KS sampling method because of the convenience, sensitivity, and patient comfort it offers.

There are certain limitations to the present study. First, this type of clinical trial requires a larger number of cases to increase the accuracy of the results. Second, although our results suggest that the KS method may improve the sensitivity of GSS over conventional swabbing, this must be confirmed by a study with randomized GSS sampling.

The KS method offers a noninvasive alternative for GSS sampling that shows higher sensitivity in the diagnosis of acute male urethritis at thresholds of both ≥5 and ≥2 PMNL/HPF compared with the conventional GSS method. Especially considering its high diagnostic sensitivity and significant agreement with PCR results in urethritis cases with low-grade inflammation and no observable discharge, the KS method could reduce the rate at which these patients are overlooked in clinical practice. However, studies with randomized sample collection are needed to verify our results.

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