



Diagnosing Herpetic Genital Ulcers in a Resource-Limited Setting- What Works?

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Authors' contributions

This work was carried out in collaboration among all authors. Author VS contributed to this study by writing the protocol of the study, collecting the specimens, performing the tests and collecting literature and study material for the study. Author SM conceived and designed the study and its protocol and reviewed the manuscript from the first draft stage to its final submission stage. Author LD was responsible for guiding the performance of HSV viral culture and PCR and also ensuring adequate resource materials for the tests. Author NK was responsible for performance of the viral culture and PCR and guiding the first author in the performance of these tests. Author VR was responsible for reviewing the manuscript and providing inputs on its draft and final versions. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Globally, viral agents, especially herpes simplex virus (HSV), have overtaken the bacterial causes of genital ulcers. Very few laboratories in India, perform culture techniques and polymerase chain reaction (PCR) for diagnosis of genital ulcers. This study aimed to establish the utility of existing tests, which are cheaper and need less technical expertise, when compared to newer tests such as PCR.

Study Design: This cross sectional study was carried out to determine the aetiology of genital ulcers, with emphasis on diagnosis of herpetic ulcers, using newer and more accurate methods of

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diagnosis and evaluating their performance by comparing against viral culture as gold standard test.

Place and Duration of the Study: The study was carried out over a period of one year in the Apex Regional Sexually Transmitted Diseases (STD) Centre at Safdarjung Hospital, New Delhi and the Department of Microbiology, AIIMS, New Delhi.

Methodology: Fifty three patients with genital ulcers were included in the study. Specimens from ulcers were taken for various tests, including Giemsa stain, ELISA for HSV-1 & 2, PCR and Viral culture for HSV.

Results: HSV was identified in 31 of 53 cases (58.5%), including 03 cases of HSV-1, and 28 cases of HSV-2. Sensitivity and specificity of PCR was 90.0% and 84.85%, respectively. Viral culture positivity was 37.7%.

Conclusion: Genital herpes is associated with an increased risk of Human Immunodeficiency Virus (HIV) acquisition, and clinical manifestations are diverse; hence a presumptive diagnosis should be confirmed by reliable laboratory tests. Nucleic acid amplification tests (NAAT) are the most sensitive methods for direct detection of HSV. The extensive validation of these tests allows for their application in routine laboratory settings with consistency and greater diagnostic accuracy. When standardised and used, PCR is a highly reproducible, rapid and labour efficient method for HSV detection.

Keywords: Genital ulcer; herpetic genital ulcer; herpes simplex virus; syndromic diagnosis; genital ulcer disease.

1. INTRODUCTION

According to the World Health Organization (WHO), sexually transmitted infections (STIs) rank among the top five disease categories for which adults seek health care [1]. In developing countries, STIs account for 10%–20% of adult patients attending government health facilities [2].

Globally, genital herpes is the commonest cause of genital ulcer disease, with an incidence of more than 20 million cases [3]. It is mainly caused by *Herpes simplex virus- 2* (HSV-2), but can also be caused by *Herpes simplex virus- 1* (HSV-1) [4].

The prevalence and aetiology of genital ulcer disease (GUD) in different geographical areas and populations in developing countries have been found to vary widely, and are also changing over time. In India, STIs pose a major public health problem. Syndromic diagnosis followed by empirical treatment is the norm and it is difficult to arrive at an aetiological diagnosis due to the lack of reliable laboratory facilities in most centres [5].

2. BACKGROUND

With the rise of human immunodeficiency virus (HIV) infection in the last 2 decades, better healthcare, advent of antibiotics and subsequent

behavioural, social and psychological changes, the aetiological spectrum of GUD has shifted from bacterial to viral STIs [6,7].

The definitive diagnosis of genital herpes relies on demonstrating the presence of HSV in the genital area, either by virus isolation or detection of antigen. Newer advances include molecular diagnostic tests like Polymerase Chain Reaction (PCR). Virus isolation in cell culture has long been considered the diagnostic gold standard for HSV [8].

There are very few laboratories in India, catering to the laboratory diagnosis of STIs, especially in the government sector. Laboratories that perform culture techniques and PCR for aetiological diagnosis of GUDs are even fewer. Most of these tests are very expensive and out of reach of the common man at this present juncture.

There are hardly any studies from India, focusing, firstly, on aetiological spectrum of GUDs, and even fewer when it comes specifically to performance of available laboratory tests [6,7].

This study attempted to determine the recent trends in the aetiology of GUDs towards herpes infection by using newer and more accurate methods of diagnosis and evaluating their performance by comparing each method against viral culture, as the gold standard test.

It also aims at establishing either the utility or the need of phasing out existing tests, which are cheaper and need less technical expertise, compared to newer tests such as PCR.

We have also studied risk factors amongst the study cohort for acquiring the disease, and HIV prevalence.

3. MATERIALS AND METHODS

A cross-sectional study was carried out in the Department of Microbiology and Apex Regional STD Centre, V.M.M.C and Safdarjung Hospital, New Delhi, and the Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India. A total of 53 consecutive attendees of the male and female STI clinic of the institution, with clinically diagnosed GUD, were included.

3.1 Inclusion Criteria

All STI Clinic attendees (both males and females) who were sexually active and presented with genital lesions/ulcers were included.

3.2 Exclusion Criteria

1. Asymptomatic patients.
2. Patients who were sexually naïve.
3. Women during menstruation presenting to STI Clinic. These patients were asked to report back after their menstrual cycle ended.
4. Patients reporting for urethral/vaginal/cervical discharge with no evidence of ulcers.
5. Patients unwilling to submit themselves for investigative procedure.

The study was submitted to the Institutional Ethics Committee (IEC) for approval before commencement of the study, and informed consent was taken from each patient.

3.3 Sample Collection

Sterile flocked nylon swabs were used to collect genital ulcer specimens from the base and edge of ulcers for smears. These were used to perform Giemsa staining, viral culture and PCR. Blood sample was collected from each patient to perform serological test (HSV ELISA) for anti-HSV IgM antibodies and also for HIV seropositivity status [in accordance with National

AIDS control organisation (NACO) guidelines for HIV testing] [9].

3.4 Methodology for Diagnostic Tests

3.4.1 Microscopy

Giemsa stained smear of ulcer scraping was prepared to look for multi-nucleated giant cells (MNGC) typical of the cytopathic changes caused by HSV, and less frequently "ground glass" appearance or Cowdry type- A inclusion bodies [10].

3.4.2 Serology

Anti HSV-2 IgM ELISA was performed using sera from blood samples:

Enzyme immune-assay (*NovaLisa™ - NovaTec Immundiagnostica GmbH - Dietzenbach, Germany*) for the qualitative determination of IgM-class antibodies against HSV-2 in human serum was carried out according to manufacturer's instructions.

3.4.3 PCR and viral culture

All the scrapings and/or swabs from genital lesions were transported immediately to Department of Microbiology, All India Institute of Medical Sciences under appropriate cold chain conditions in viral transport medium. The swabs, collected in viral transport medium, were kept at 4°C overnight after adding antibiotics (Penicillin=100U/ml and Streptomycin=100 µg/ml). The next day, specimens were vortexed for 15 seconds for removing the cells from swabs and homogenization was achieved. These samples were thereafter centrifuged at 2000-2500 rpm for 10-15 min. The supernatant was aliquoted into two separate 1.5 ml micro-centrifuge tubes for virus isolation and PCR, respectively, and stored at -80°C.

DNA was extracted for PCR using QI Aamp Viral RNA Mini Kit (QIAGEN, Hamburg, Germany) as per manufacturers' instruction. All the samples were tested with conventional PCR methods. One upstream primer and two type-specific downstream primers were prepared to amplify DNA from the HSV type 1 and type 2 DNA polymerase gene [11].

Primers:

DNA polymerase gene (Gene bank accession no- X04771)

HSV (nucleotide sequence no. 1558–1579) -
5'ATG GTG AAC ATC GAC ATG TAC GG-3'

HSV-1 (nucleotide sequence no. 2004–2026) -
5'CCT CGC GTT CGT CCT CGT CCT CC -3'

HSV-2 (nucleotide sequence) - 5'CCT CCT
TGT CGA GGC CCC GAA AC-3'

3.4.4 Three-step protocol of thermal cycling

2 min at 94°C for one cycle and then 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C for 45 cycles.

Post amplification detection was done by electrophoresis in 2% agarose gel and visualized in Gel Doc XR+ gel documentation system (*Bio-rad laboratories, Inc. USA*). Using these three primers simultaneously in the PCR reaction mixtures, both types of HSV DNA were amplified to produce products of different sizes. By direct gel analysis, the products of standard HSV type 1 and type 2 strains had the predictive sizes of 469 and 391 base pairs, respectively.

For **viral culture**, Vero cell line obtained from *National Centre for Cell Sciences (NCCS), Pune, India* was used. Cell line was grown in Minimum Essential Medium (MEM), Eagle with 10% Fetal Calf Serum, 0.292 gm/L of L-glutamine (*Sigma Aldrich Corp, USA*), and antibiotics (100 U/ml Penicillin and 100 µg/ml Streptomycin) at 37°C. Standard strains of HSV-1 and HSV-2 obtained from *National Institute of Virology, (NIV, Pune, India)* were used as positive controls with each batch of virus isolation.

After appropriate preparation of the clinical sample, the processed inoculum (0.2 ml) was added to the cell culture tube. Cell culture maintenance medium (with 2% Fetal Calf Serum) was added. Inoculated cell culture tubes were incubated at 35°C, examined daily under inverted microscope for typical CPE (rounding, ballooning and syncytia formation). Tubes, not showing CPE were incubated for a period of seven days because HSV proliferates rapidly. One tube of each specimen, (whether or not showing any CPE) was frozen at -70°C. Cells from the other tube with CPE (>75%) were harvested and 2 cell spots were made on the slide for each sample. The slides were fixed with chilled acetone at -20°C for half an hour and stored at -20°C till direct immunofluorescence staining (DFA) was done to confirm the presence of virus. A blinded second passage was done for

all the samples showing either no CPE or CPE in <75% area of cell monolayer. For DFA, *Light Diagnostics™ SimulFluor® HSV 1/2 Immunofluorescence Assay* was used as per manufacturer's instructions and appropriate controls were set up. Mounting was done under a coverslip using an aqueous Mounting Medium pH 8.5 and slides were visualized under epifluorescent microscope (*Nikon Eclipse, Japan*), immediately after preparation, at 100-200x for cells exhibiting fluorescence and then at 400x for characterization of fluorescence.

An HSV-1 positive reaction was indicated by bright apple-green fluorescence in the cytoplasm and nucleus of the infected cell. An HSV-2 positive reaction was indicated by a yellow-gold fluorescence in the cytoplasm and/or cell membrane of the infected cells. The negative cells stained red due to Evan's blue counterstain.

All data was classified and entered in a tabular form. Statistical analysis was performed using SPSS version 20.0. The clinical profile of patients was analyzed by Chi-square test for qualitative variables. Student t-test was performed for comparison of quantitative variables. A 5% probability level was considered as statistically significant i.e., P= 0.05.

Sensitivity, specificity, positive predictive value and negative predictive value of each laboratory method was calculated and compared.

4. RESULTS AND DISCUSSION

Genital herpes is a preventable chronic disease and there is a looming threat of an epidemic in developing countries. It is the commonest cause for not just genital ulcer disease but all STIs in general, replacing bacterial aetiologies, in the developing countries [12,13].

The demographic profile of the present study shows that the vast majority of patients were males (84.9%). The mean age was found to be 33.18 years. This is in accordance with the age group and gender patterns seen in other studies from Delhi [5,6,14]. In all these studies including ours, the number of women attending STI clinics is dismally low and this cannot be attributed just to the fact that herpes genitalis is largely asymptomatic in women [15]. Many cultural factors come into play too, as shown in a study by Ragi Ravi et al. in 2011, who showed that social stigma, gender discrimination, illiteracy associated with poverty, lack of healthcare, low awareness and media exposure and low

autonomy contribute to the veiled culture among rural women [16].

The demographic profile of the 53 study patients was as follows:

Forty-five (84.9%) subjects were male and only 8 (15.1%) were female. Mean age of patients was 33.18 years.

Sixty-six percent (35) of the respondents were married, 32.1% (17) were unmarried, while 1 patient was divorced.

On evaluation of high risk behaviour amongst the study group, the following observations were made:

Mean age at first sexual encounter was found to be 20.4 years.

A total of 31 subjects (58.5%) out of the entire cohort had multiple (2 or more) sexual partners during their lifetime. The exposure of study participants to current sex partners was assessed.

There were 12 patients who had non regular sexual partners. Out of the 24 cases, who had a regular partner (RP), 7 also had a non-regular partner (NRP) concurrently, while 3 were also exposed to commercial sex workers (CSW) as can be seen from the Table 1.

In this study, 43.4% of the participants presented with recurrent episodes of GUD (two or more episodes).

The prevalence of HIV was 11.3% among our study population, which is more than the NACO

2011 surveillance report (5.2% HIV prevalence in STI clinic attendees in Delhi). This is in keeping with a large body of evidence suggesting an elevated risk of HIV transmission among individuals with GUD, and particularly amongst those with HSV-2 [12,13].

HSV was identified in 31 out of 53 (58.5%) of GUD cases, which included 03 cases (9.6%) of HSV-1, and 28 cases (90.3%) of HSV-2 positivity.

The performance of various diagnostic tests was observed as in Table 2.

The sensitivity of Tzanck smear, in our study, was 60% and specificity was 81.8%. It was also seen that detection was enhanced in patients who presented with first episode of genital herpes, with 10 cases positive versus 8 positive when patients presented at or after the second episode.

The results observed for HSV IgM ELISA did not validate the efficiency of the test for diagnosis. The positivity rate was only 28.3%. Sensitivity and specificity was 30% and 72.73% respectively. No significant difference in positivity rates was found with respect to the number of episodes.

Sensitivity and specificity of PCR was found to be 90.0% and 84.85% respectively. PCR was positive in 10 patients with first episode of GUD (18.9%) and in 13 patients (24.5%) with 2 or more episodes.

Table 1. Sexual behaviour of study participants

Current sex partner	Frequency	Percentage
None	1	1.9%
Regular partner (RP)	24	45.3%
Non-regular partner (NRP)	12	22.6%
RP + NRP	7	13.2%
Commercial sex worker (CSW)	6	11.3%
RP + CSW	3	5.6%
Total	53	100%

Table 2. Diagnostic tests performed for genital ulcers

Diagnostic test	Positivity rate (%)
Tzanck Smear for MNGC	34.0
HSV-2 IgM ELISA	28.3
HSV PCR	43.4
HSV Culture	37.7

Viral culture was positive in 10 out of 20 patients and 7 out of 8 patients in 1st and 2nd episode respectively. However, only 3 of the 12 cases were culture positive when the patients presented with greater than 2 episodes (P value = .005).

Of the 30 patients who presented with first episode of genital ulcers, both PCR and culture had similar detection rates and were positive in 10 cases (18.9%). With the number of episodes increasing, the positivity rate of viral culture declined significantly. In cases with more than 2 episodes of genital infection, PCR was positive in 28.3% whereas culture was positive in only 5.7% (p-value= 0.022). (Table 3) (Fig. 1).

The mean age of participants of this study at first sexual encounter was found to be about 20 years for both the genders. Early age at first sexual experience is a risk factor for acquiring sexually transmitted infections like genital herpes as was shown in the study on HIV positive individuals in Delhi, by Karad AB et al. [17]. They found that in patients positive for HSV-2 serology, 84.1% had their first sexual experience at less than 19 years of age [18].

In this study, more than half (58.5%) of the study participants, had sexual contact with more than one partner (35.8% had sex with non-regular partner and/or a regular partner, 16.9% had sex with a commercial sex worker). All these participants were males. Considering the conservative patriarchal nature of many Indian societies, it is understandable that GUDs are more common among males because they have more liberties and opportunities to practice high risk behavior.

We detected the prevalence rate of HIV as 11.3% among our study population, which is more than the NACO 2011 surveillance report (5.2% HIV prevalence in STI clinic attendees in Delhi). This is in keeping with a large body of evidence suggesting an elevated risk of HIV transmission among individuals with GUD, and particularly among those with HSV-2 infection [19,20]. Of the 6 HIV seropositive cases presenting with genital ulcers, 5 were diagnosed with herpetic GUD. However, in one of the cases, only Tzanck test was positive, which may be a subjective error. The use of acyclovir for herpetic GUDs has shown remarkable benefit in terms of reduction in HIV transmission [21].

Table 3. Comparative performance of diagnostic tests vs viral culture

Diagnostic test	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Measurement of agreement (kappa value)
Tzanck Smear	60.0	81.8	66.7	0.427
HSV-2 IgM ELISA	30.0	72.7	40.0	0.029
HSV-PCR	90.0	84.8	78.3	0.727

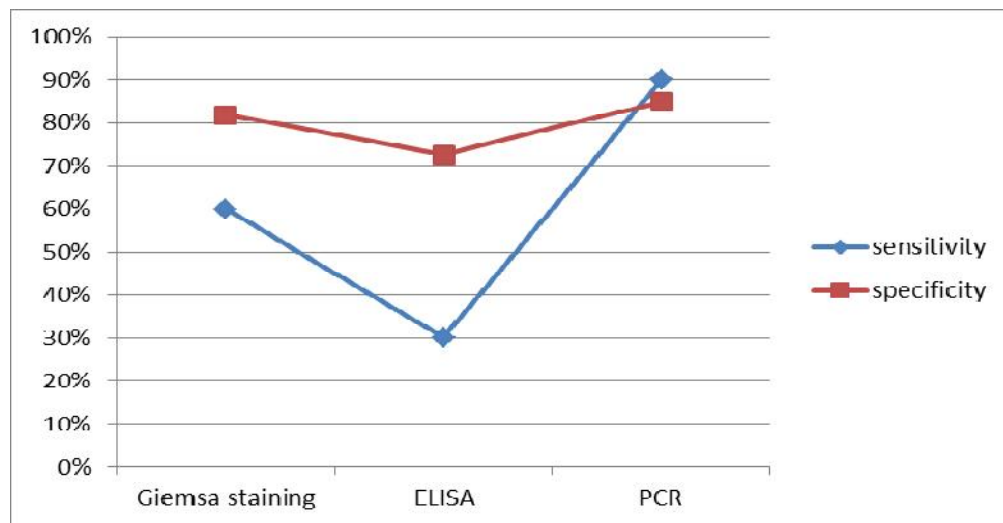


Fig. 1. Comparison of sensitivity and specificity of diagnostic tests

HSV was identified in 31 (58.5%) out of 53 GUD cases. Aetiology could not be determined for the rest of the cases except in 2 cases (3.77%) of primary chancre of syphilis which were diagnosed based on dark field microscopy and VDRL test. This is in agreement with other studies in both the developed⁴ and developing countries, like Brazil [20] and India [6,22].

HSV-1 positivity has ranged from 3.2% (Gomes Naveca F et al. 2013, Brazil) [20] to 7.6% (Risbud et al. 1999, India) [19] and 32.2% (Muralidhar S, et al. 2013, New Delhi, India) [6] in various studies indicating the existence of geographical variations.

For the laboratory diagnosis of genital herpes, Tzanck test is a simple and inexpensive albeit less sensitive test [10]. However, it is a useful tool in many resource poor settings of our country.

The sensitivity of Tzanck test, from genital ulcer scrapings in our study, was 60% and specificity was 81.8%. It was also seen that positivity was higher in patients who presented with first episode of genital herpes as has been observed in previous literature [23].

The results observed for HSV IgM ELISA did not validate it as an efficient diagnostic test. The positivity rate was only 28.3%. Sensitivity and specificity was 30% and 72.73% respectively. In the previous study done by Muralidhar et al. at the same centre, the positivity rate was only 4.54% [6]. This variation in performance of ELISA may be due to various factors like: Inherent efficacy of the particular brand of ELISA kit and the natural history and progression of the disease itself. Immunoassays depend on HSV antibodies and so the sensitivities of these tests are affected by the time elapsed since initial infection. Detection is optimal when the test is conducted a minimum of 21 days after initial infection and may improve if the test is run >40

days after first episode [24]. Also, as this is HSV-2 IgM ELISA, the test failed to detect antibodies in three patients with HSV-1 genital infection. Hence these were classified as 'false-negatives'.

HSV IgM antibodies appear initially with primary infections and levels start to decline within 2-3 months, reappearing sporadically when there are recurrences. Thus, detection of IgM antibodies does not indicate recently acquired infection reliably, and primary infection cannot be distinguished from a recurrent episode by this method [25].

Virus isolation is the traditional gold standard for HSV detection and the reference method against which all other tests are measured [26,27]. Diagnosis of HSV infection with tissue culture has low sensitivity because HSV is isolated from lesions in about 80% of primary infections but in only 25–50% of recurrent lesions [14], and in even fewer people whose lesions have begun to heal [28]. Also cell culture is technically demanding and time consuming.

As shown in Table 4, the HSV virus isolation rate from genital ulcer lesions has varied from 8% to 37.3% in various studies. Nucleic acid amplification based methods (PCR or real time PCR) have higher rate of detection as compared to cell culture. The enhanced sensitivity ensures that even lesion samples containing minimal cells can be analyzed with good sensitivity. The nucleic acid tests are much less affected by specimen storage beyond 48 hours, freezing, thawing or bacterial contamination.

Literature pegs the sensitivity and specificity of nucleic acid amplification tests (NAAT) as more than 90% [27,29-32]. The sensitivity of PCR in our study was also 90% although specificity is lower at 84.85%. This could be attributed to two false-negative results, sample transport, contamination or procedural errors.

Table 4. Virus isolation rate for diagnosis of muco-cutaneous herpes simplex virus (HSV) infection in various studies

Studies	Number of samples tested from cases with GUD	Virus isolation (% positive for HSV)
Scoular A, et al. 2002 [29]	236	37.3%
Espy MJ, et al. 2001 [30]	198	33.8%
Wald A, et al. 2003 [27]	4670	12.0%
Ramaswamy M, et al. 2004 [31]	233	34.0%
Goyal K, et al. 2013 [32]	25	8.0%
Gitman MR, et al. 2013 [33]	171	29.8%
Present study	53	37.7%

In our study, the detection rate of conventional PCR was 43.4% which is similar to that shown by Waldhuber MG, et al. (46%) [34]. Detection was higher in first episode infections when compared to recurrent infections, and this was statistically significant.

There were two samples, positive by culture but negative by conventional PCR. We believe that these 2 samples were actually false negative by conventional PCR. The analysis of the two apparently false-negative samples is interesting. There are many factors known to cause false-negative PCR results. Nucleic acid extraction, transport and quality of samples can lead to false negative PCR results. Inhibitors of *Taq* polymerase can be found in clinical specimens and serum, which can ooze out as the vesicle ruptures or if vigorous rubbing is done for sample collection. This could result in false-negative results for PCR. Sequence variability is an alternative source for potential false-negative PCR results.

The advent of real-time PCR systems, where products are detected in a closed-tube system without any post-amplification handling, has minimized the risk of false-positive results by PCR. Real time PCR assay has many other advantages also over regular PCR as the detection can be done in around 5-6 hours beginning from the extraction of nucleic acid from the clinical sample till the setting of the assay and final analysis of the results.

5. CONCLUSION

NAATs are currently the most sensitive methods for the direct detection of HSV [27]. The availability of these tests from commercial sources and their extensive validation allows for the application of this technology in routine clinical laboratory settings with consistency and greater diagnostic accuracy.

Although most HSV infections are subclinical, clinical disease can be associated with substantial physical and psycho-social morbidity. In addition, genital herpes is associated with an increased risk of HIV acquisition. The clinical manifestations are diverse; hence a presumptive diagnosis of HSV should be confirmed by laboratory tests.

After assessment of all the diagnostic tests available in our setting, it was seen that the PCR assay is the test of choice for the diagnosis of

genital herpes in symptomatic patients presenting with lesions. The test is the most sensitive and specific of all the methodologies used in this study.

Use of PCR in routine diagnostic settings is limited by concerns over its cost and contamination. On a per-specimen basis, the cost of PCR is more than HSV culture isolation. However, capital costs of tissue culture are greater in the long term [27]. Most importantly, the positivity rate for virus detection by PCR is 4 times greater and the results are more reliable [27]. So, when standardised and used, it is a highly reproducible, rapid, and labour efficient method for HSV detection from genital swabs.

Also, PCR allows not only detection of HSV DNA, but also differentiation between HSV-1 and HSV-2 genotypes.

The major limitation of the present study is its small sample size, as large prospective studies would certainly throw more light on the constraints in diagnosis of genital ulcers in resource limited settings. However, this study has highlighted some very important aspects of diagnosing genital ulcer diseases in an accurate manner. It is hoped to chart a course towards replacing conventional diagnostic methods by PCR, which is considered as the "new gold standard" in diagnosis of genital herpes. Also, the study will provide an evidence based impetus for an effective implementation of STI programs in India.

CONSENT AND ETHICAL APPROVAL

Institutional ethics clearance and informed consent were taken before conducting the study, and appropriate statistical analysis was performed.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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