DOI: 10.7860/NJLM/2024/62892.2860

Original Article

Microbiology Section

Laboratory Diagnosis of Herpes Simplex Virus in Mucocutaneous Lesions by Light Microscopy, ELISA and PCR: A Cross-sectional Study from a Tertiary Care Hospital

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ABSTRACT

Introduction: Herpes Simplex Virus-1 (HSV-1) and HSV-2 can produce mucocutaneous lesions and Central Nervous System (CNS) infections, some of which may be life-threatening. There are no known animal vectors for HSV, and although experimental animals are easily infected, humans appear to be the only natural reservoir. Herpes infection is the predominant cause of genital ulcers worldwide. An increase in Polymerase Chain Reaction (PCR) usage for HSV detection and an increase in HSV-2 reactivation frequency among HSV/Human Immunodeficiency Virus (HIV)-co-infected persons can be attributed to this.

Aim: To detect HSV infection by light microscopy, Enzyme Linked Immuno Sorbent Assay (ELISA), PCR in clinically suspected cases and to assess the utility value of PCR over ELISA and light microscopy.

Materials and Methods: A cross-sectional study was conducted on 84 samples collected from clinically suspected cases of herpes simplex infections at the Department of Dermatology and Sexually Transmitted Diseases (STD) Clinic. The collected samples were processed in the Department of Microbiology, Bangalore Medical College and Research Institute, Bengaluru, Karnataka, India during the period from November 2018 to May 2020. Swabs collected from the patients were processed for investigations including light microscopy, HSV-1, HSV&2 PCR. Blood samples were collected from those patients for HSV1+2 IgM ELISA. Descriptive statistics such as mean and Standard Deviation (SD) for continuous variables, and frequencies and percentages for categorical variables were calculated.

Results: Total of 84 cases of HSV were included 68 (81%) were with suspected cases of herpes genitalis, and 16 (19%) presented with herpes labialis. The prevalence of HSV infection was found to be 59.5%. In the case of herpes genitalis and herpes labialis, the majority were in the age group 31-40 years. Males were more affected compared to females in the case of herpes genitalis, while females were more affected in the case of herpes labialis. PCR had a sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) of 74%, 100%, 100%, and 72.3%, respectively.

Conclusion: PCR can be used as a gold standard test as well as a confirmatory test when compared to IgM ELISA and Tzanck smear for HSV diagnosis.

Keywords: Herpes genitalis, Herpes labialis, IgM enzyme-linked immunosorbent assay, Polymerase chain reaction, Tzanck smear

INTRODUCTION

Sexually Transmitted Infections (STIs) are infections that spread through sexual intercourse [1]. There are over 30 infections that can be transmitted sexually [2]. Various aetiological agents cause STIs, including bacterial agents such as *Chlamydia trachomatis* (Lymphogranuloma venereum), *Neisseria gonorrhoeae* (Gonorrhea), *Haemophilus ducreyi* (chancroid), *Treponema pallidum* (syphilis), *Gardnerella vaginalis* (vaginosis), *Klebsiella granulomatis* (Granuloma inguinale), and viral agents such as HSV (Genital herpes), HIV, Cytomegalovirus, HBV, Molluscum contagiosum (venereal wart), and Human papillomavirus (Genital warts). Fungal agents such as *Candida albicans* (vaginal thrush) and parasitic agents include *Trichomonas vaginalis* (vaginitis, urethritis). HSV occurs naturally only in humans. The virus has two types: HSV-1 and HSV-2 [1]. The prevalence of HSV type 2 infections in the general population ranges from 10-60%, and genital herpes is the main cause of genital ulcers worldwide. HSV-2 mainly causes herpes genitalis. There is a much higher risk of HIV acquisition in HSV infection. Virological and type-specific serological tests should be used routinely as clinical diagnosis is neither sensitive nor specific [3].

Light microscopy has the advantage of being inexpensive, rapid, and simple to perform. The drawback of light microscopy is a lack of specificity, and sensitivity will depend on the stage of the lesion. IgM ELISA can be utilised to discriminate between recent and chronic infection [4]. PCR was shown to increase the overall rate of HSV detection by 61-71%. Even in patients presenting with visible genital ulcerations, PCR detected 88% more infections than virus culture [5].

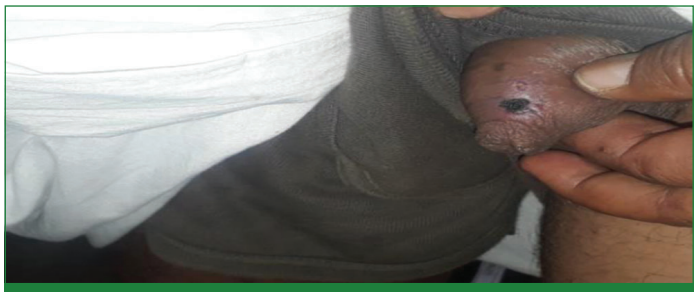
The uniqueness of this study lies in using conventional PCR in clinically suspected cases of HSV infection, which is cost-effective compared to other studies that use different diagnostic methods such as real-time PCR. The effectiveness of treatment for HSV infections depends on the rapid administration of appropriate antivirals. This creates the need to establish a prompt diagnosis and necessitates HSV diagnostic testing that is both rapid and sensitive [6-9]. This necessitates the usage of a good diagnostic tool. PCR is considered the gold standard method compared to IgM ELISA and light microscopy [10]. Therefore, this study highlights the importance of PCR over ELISA and light microscopy in the diagnosis of HSV infection. The aim of the present study was to detect HSV infection by light microscopy, ELISA, PCR in clinically suspected cases and to assess the utility value of PCR over ELISA and light microscopy.

MATERIALS AND METHODS

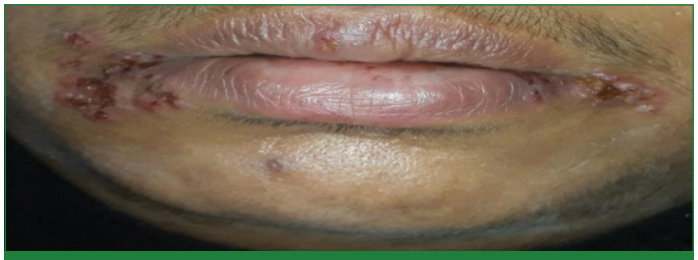
A cross-sectional study was conducted on 84 samples collected from clinically suspected cases of herpes simplex infections at the Department of Dermatology and STD Clinic. The collected samples

were processed in the Department of Microbiology, Bangalore Medical College and Research Institute, Bengaluru, Karnataka, India during the period from November 2018 to May 2020. Ethical committee approval (IEC Approval number): BMC/PG/124/2018-19 and consent was obtained for the study.

Inclusion criteria: Samples were collected from all vesiculoulcerative lesions from clinically suspected herpes simplex infection, including immunocompetent individuals and various age groups as shown in [Table/Fig-1,2].



[Table/Fig-1]: Lesion of herpes genitalis.



[Table/Fig-2]: Lesion of herpes labialis.

Exclusion criteria: Patients who are on antiviral therapy for HIV and other bacterial STI infections such as syphilis were excluded from the study.

Study Procedure

Sample collection: Samples were collected from all clinically suspected cases of HSV infection.

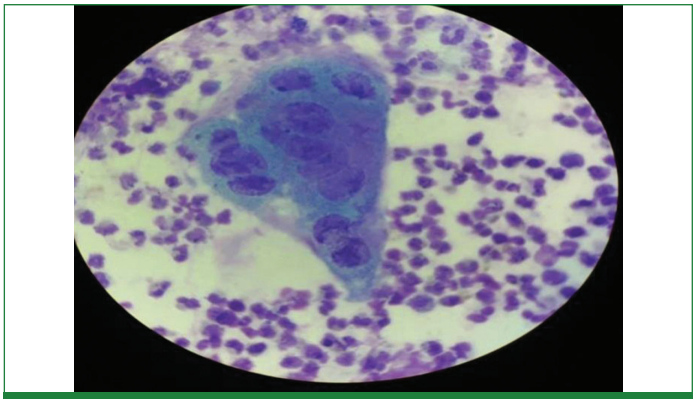
- **Conventional PCR:** Conventional PCR for skin and genital lesions: Samples were collected from the base of the vesicle using cotton wool swabs or Dacron swabs, and transported into Viral Transport Medium (VTM) and immediately taken to the laboratory [10].
- **IgM ELISA:** A blood volume of 10 mL was collected aseptically from all patients into plain tubes. The sera were separated using a centrifuge (1000x) for 10 minutes and transferred to the microcentrifuge tubes using a pipette, and stored at -20°C for one week [11].
- **Light microscopy:** The intact roof of the young vesicle or bulla was incised with a scalpel, and the contents were spread onto a clean microscopic slide. It was then stained with Giemsa stain and allowed to dry. The slide was then examined under a light microscope for a tzanck cell, which is a keratinocyte with a hypertrophic nucleus as shown in [Table/Fig-3] [10].
- **Serological method:** IgM ELISA was performed with serum specimens [12].

Sample size: Based on a previous study, PCR for HSV was positive for about 82% of cases [13]. The sample size calculation is as follows:

Sample size (n)=Z^2pq/d^2

where z score (Z)=1.96, prevalence p=82, q=(100-P)=18, and margin of error (d)=8.2.

Therefore, the sample size (n)=$(1.96)^2\times82\times18/(8.2)^2$=84. The sample size was 84.



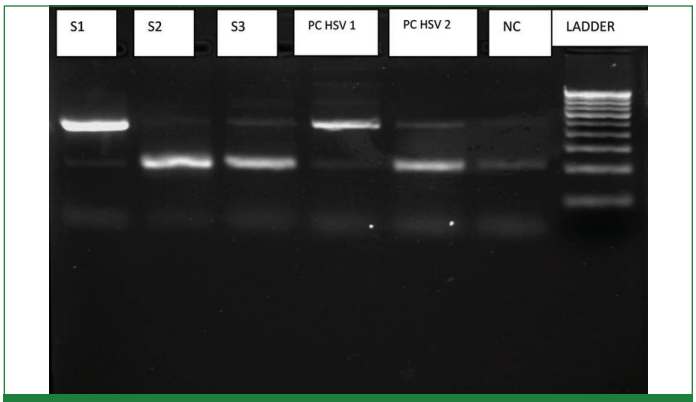
[Table/Fig-3]: Tzanck smear stained with Giemsa stain showing multinucleate giant cell under oil immersion (100x).

IgM ELISA: Principle of the Assay

IgM antibodies in the sample are first captured by the solid phase coated with anti-human IgM antibody. After washing out all other components of the sample, particularly IgG antibodies, the specific IgM captured on the solid phase is detected by the addition of a purified preparation of inactivated HSV 1 and 2 labelled with a specific antibody conjugated with peroxidase Horse Radish Peroxidase (HRP). After incubation, microwells are washed to remove unbound conjugate, and then the chromogen/substrate is added. In the presence of bound conjugate, the colourless substrate is hydrolysed to a coloured end product, whose optical density may be detected and is proportional to the amount of IgM antibodies to HSV 1 and 2 present in the sample [12,14].

Molecular Method

- **DNA extraction:** Deoxyribonucleic Acid (DNA) was extracted from the swabs collected in VTM using the DNA mini kit (Qiagen) according to the manufacturer's instructions. Nucleic acid amplification was performed using the DNA extracted from the sample [13].
- **Polymerase Chain Reaction (PCR):** The PCR reaction contained 25 µL of PCR master mix, two microliters of forward and reverse primers each, eight µL of DNA extract, and 7.5 µL of deionised nuclease-free water to obtain a final volume of 50 µL. PCR amplification was be carried out. It runs for 35 cycles with a final extension at 72°C for five minutes. The PCR products are electrophoresed and visualised on 2% agarose. Bands were visualised under Ultraviolet (UV) light with a wavelength of 450 nm. Known HSV-1 and HSV-2 DNA were used as positive controls, and DNA negative for HSV-1 and HSV-2 was used as negative controls. Amplicons will be identified and differentiated as follows: HSV-1 gG PCR amplicon size 487 bp and HSV-2 gG PCR amplicon size 214 bp, as shown in [Table/Fig-4] [13].



[Table/Fig-4]: Gel showing the bands of HSV.
S1-S3 Samples, PC HSV 1: Positive control for HSV-1, PC HSV 2: Positive control for HSV-2, NC: Negative control

The forward and reverse primer sequences are shown in [Table/Fig-5] [13].

Primer	Primer sequence (5'-3')	Product size (bp)
HSV 1 gG Forward	CCCCCATGCCAAGTATTGGA	487 bp
HSV 1 gG Reverse	AGACATACGTAACGCACGCT	
HSV 2 gG Forward	AGCTCCCGCTAAGGACATG	214 bp
HSV 2 gG Reverse	AGACATACGTAACGCACGCT	

[Table/Fig-5]: Primer sequences used in HSV PCR.

STATISTICAL ANALYSIS

The data was entered into Microsoft Excel (Windows 7; Version 2007), and the analysis was conducted using the Statistical Package for the Social Sciences (SPSS) for Windows software (version 22.0; SPSS Inc, Chicago). Descriptive statistics such as mean and SD for continuous variables, as well as frequencies and percentages for categorical variables, were determined. Sensitivity, specificity, PPV, and NPV were calculated. The Chi-square test was used to determine associations between categorical variables. The level of significance considered was <0.05.

RESULTS

Out of 84 clinically suspected herpes simplex cases, 68 (81%) presented with herpes genitalis, and 16 (19%) presented with herpes labialis. Among these clinically suspected cases, the majority were in the age group 31-40 years, while the fewest cases were seen among those over 60 years and in the 51-60 years age group for herpes genitalis and herpes labialis cases, respectively, as shown in [Table/Fig-6].

Age (in years)	Herpes genitalis 68 (%)	Herpes labialis 16 (%)	n (%)
≤30	18 (26.5%)	4 (25.0%)	22 (26.2%)
31-40	19 (27.9%)	8 (50.0%)	27 (32.1%)
41-50	17 (25.0%)	2 (12.5%)	19 (22.6%)
51-60	12 (17.7%)	0	12 (14.3%)
>60	2 (2.9%)	2 (12.5%)	4 (4.8%)
Mean±SD	39.94±12.12	36.63±13.75	39.31±12.43
Range			19-67

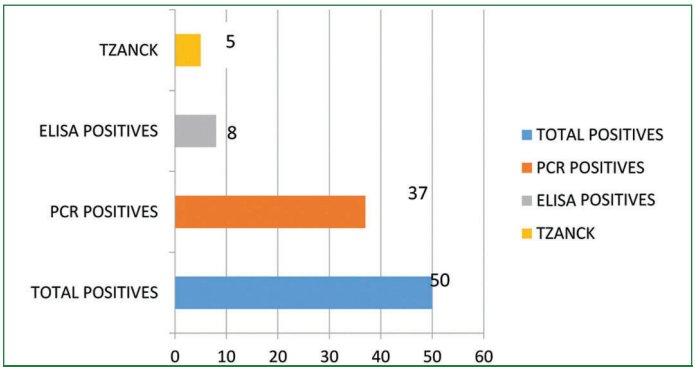
[Table/Fig-6]: Distribution of study subjects according to the age group (N=84).

The male population was more affected by genital herpes, while females are more affected by oral herpes, as shown in [Table/Fig-7].

Out of 68 suspected cases of genital herpes, 36 cases tested positive through HSV investigations such as light microscopy, HSV ELISA, and HSV PCR. Among herpes labialis cases, 14 out of 16 tested positive. Among the total 50 (59.5%) positive cases, 37 were detected by PCR, followed by IgM ELISA and Tzanck smear, as shown in [Table/Fig-8].

Gender	Herpes genitalis	Herpes labialis	Total positives (n=50)
Female	4	13	17
Male	32	1	33
Total	36 (72%)	14 (28%)	50

[Table/Fig-7]: Distribution of HSV-positive cases according to sex (N=50).



[Table/Fig-8]: HSV positives by PCR, IgM ELISA, Tzanck smear.

[Table/Fig-9] shows that HSV infection was found to be more prevalent in males and in the age group 31-40 years. It was predominant in the form of herpes genitalis, with the duration of illness around the 5th day found to be statistically significant (p-value <0.05). Ulcerative lesions were not found to be significant (p-value >0.5) among HSV positives.

Character	HSV positive (N=50) %	HSV negative (N=34) %	Chi-square	p-value
Sex				
Male	33 (66)	31 (91)	7.0714	0.007832
Female	17 (34)	3 (9)		
Age (years)				
≤30	11 (22)	11 (32.3)	16.2273	0.002729
31-40	23 (46)	4 (11.7)		
41-50	12 (24)	7 (20.5)		
51-60	3 (6)	9 (26.5)		
>60	1 (2)	3 (9)		
Duration of illness				
3rd day	10 (20)	10 (29.4)	13.7158	0.003319
5th day	26 (52)	5 (14.7)		
7th day	12 (24)	13 (38.2)		
7-10 days	2 (4)	6 (17.6)		
Ulcer				
Genital ulcer	37 (74)	32 (94)	6.4206	0.01128
Oral ulcer	13 (26)	2 (6)		
Type of lesion				
Ulcerative	48 (96)	32 (94)	0.2263	0.893017
Vesicular	1 (2)	1 (3)		
Crusted	1 (2)	1 (3)		

[Table/Fig-9]: The demographic and clinical profile of patients with HSV. (Chi-square test with level of significance p<0.05)

[Table/Fig-10] HSV-1 was present in the majority of herpes labialis cases, while HSV-2 is present in herpes genitalis cases. HSV-1 PCR positives were found to be more prevalent in cases of herpes labialis and were also determined to be statistically significant (p-value <0.05) as shown in [Table/Fig-11]. Similarly, HSV-2 PCR positives were found to be more prevalent in cases of herpes genitalis and were also found to be statistically significant (p-value <0.05) as shown in [Table/Fig-12].

PCR	Herpes genitalis	Herpes labialis	Total positives (%)
HSV-1	7 (38.9%)	11 (61.1%)	18 (49%)
HSV-2	19 (100%)	0	19 (51%)
Total	26 (70.3%)	11 (29.7%)	37 (100%)

[Table/Fig-10]: Distribution of PCR positives.

Character	HSV 1 PCR positive (18)	%	HSV 1 PCR negative (19)	%	Chi-square	p-value
Herpes genitalis	7	38.9	17	89.5	10.378	0.001275
Herpes labialis	11	61.1	2	10.5		

[Table/Fig-11]: Association of genital herpes and herpes labialis with HSV1 PCR. (Chi-square test with level of significance p<0.05)

Character	HSV 2 PCR positive (19)	%	HSV 2 PCR negative (18)	%	Chi-square	p-value
Herpes genitalis	19	100	11	61.1	9.1130	0.0025
Herpes labialis	0	0	7	38.9		

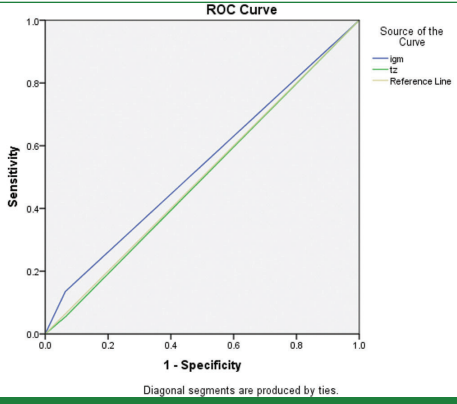
[Table/Fig-12]: Association of genital herpes and Herpes labialis with HSV2 PCR. (Chi-square test with level of significance p<0.05)

The sensitivity and NPV for HSV PCR were higher than those for IgM ELISA and Tzanck smear. The specificity and PPV were 100% for HSV PCR, IgM ELISA, and Tzanck [Table/Fig-13]. The ROC curve

illustrates the sensitivity and specificity values of the diagnostic tests with PCR as the gold standard. It indicates that IgM ELISA can be used as a diagnostic method, as the area under the curve is 0.5, while the Tzanck smear cannot be used, as the area under the curve is <0.5 [Table/Fig-14].

Stastistical parameters	HSV PCR	IgM ELISA	TZANCK Smear
Sensitivity	74%	16%	10%
Specificity	100%	100%	100%
Positive Predictive Value (PPV)	100%	100%	100%
Negative Predictive Value (NPV)	72.3%	44%	43%

[Table/Fig-13]: Statistical parameters of HSV PCR, IgM ELISA, and TZANCK smear.



[Table/Fig-14]: ROC curve with sensitivity and specificity values.

DISCUSSION

HSV affects 60-95% of the world's adult population. HSV-1 is associated with ocular, oropharyngeal, and CNS infections, while HSV-2 is mainly associated with infections of the anogenital region. HSV is the main cause of genital ulcer disease. Confirmation of the clinical diagnosis by laboratory investigations is mandatory. Laboratory investigations, such as direct tests for viral isolation, detection of antigen, and molecular methods, are crucial. PCR is the most sensitive method for detecting HSV types 1 and 2. It is more sensitive than cell culture for detecting the herpes virus [15]. The present study aimed to diagnose HSV infection using light microscopy, ELISA, and PCR. In this study, samples were collected from 84 clinically suspected herpes simplex cases, with herpes genitalis and herpes labialis being the majority, with age group of 31-40 years being most affected, comparable to studies conducted by Mathew R et al., which showed the major affected age group was between 20-40 years [15]. Wald A et al., found that the median age group affected was 34 years [16]. Langenberg AG et al., showed the affected age group was less than 30 years [17]. Amudha VP et al., found the age group to be between 20-60 years [18]. Herpes genitalis was more common among males compared to females in the present study, which was comparable to Wald A et al., Langenberg AG et al., Amudha VP et al., and Aggarwal A and Kaur R [16-19]. The present study's prevalence was 59.5%, comparable to Smith JS and Robinson NJ [20]. Among the 84 suspected herpes simplex cases, 50 (59.5%) tested positive through one of the tests performed. Among the 50 cases of HSV infection, 74% had genital ulcers [Table/Fig-9], comparable to the study conducted by Filen F et al., [21]. Oral ulcers constituted about 26%, which was comparable to Abraham AM et al., [22]. The prevalence of HSV-1 and 2 in herpes genitalis and herpes labialis in the present study was 72% and 28%, respectively, and was comparable to studies conducted by Slomka MJ and Sridharan G et al., [23,24]. The prevalence of HSV-1 in herpes labialis in the present study was 61.1% [Table/Fig-10], comparable to the study conducted by Abraham AM et al., and HSV-2 prevalence is comparable to the study conducted by Schremser V et al., [22,25].

Virus culture has been the most common method for detecting mucocutaneous herpes virus infections, considered the "gold standard." However, virus isolation is slow and labor-intensive. In recent years, PCR has gained acceptance as a reference method when rapid, sensitive, and specific diagnosis is crucial. PCR has demonstrated higher sensitivity than culture, in agreement with studies of HSV detection in dermal, genital, ocular, mouth, and skin swabs from adults [26]. Among nucleic acid amplification techniques, particularly PCR has demonstrated superior sensitivity to all other diagnostic methods for the detection of HSV infections, prompting routine implementation in clinical laboratories [27].

PCR diagnosis of HSV infection is a major advancement in the use of real-time PCR for detection and quantification. Amplification of the target DNA and hybridisation to the subtyping specific fluorescent probes are conducted in a single PCR, minimising the chances of possible contamination. In detecting asymptomatic shedding or shedding episodes in the absence of clinically obvious lesions, real-time PCR has also been proven to be more sensitive [28]. Quantitative PCR for HSV DNA appears to be highly accurate [26]. Studies conducted by Mathew R et al., and Mackay IM et al., showed that antiviral drugs might have decreased viral shedding in patients and could have been responsible for the reduced number of HSV detections [15,29]. In present study, the low sensitivity of HSV PCR compared to studies conducted by Van Doornum GJ et al., Gitman RM et al., and Gardella C et al., can be attributed to the conventional PCR method used as well as antiviral therapy administration [30-32]. The present study was comparable to Mackay MI et al., and Dominguez SR et al., [29,33]. HSV PCR demonstrated 100% specificity, 74% sensitivity, 100% PPV, and 72.3% NPV. Based on these results, PCR is considered the gold standard compared to IgM ELISA and Tzanck smear.

IgM antibodies start to develop up to 10 days from exposure and can last up to 7-10 days. Test positivity will depend on this time interval for the formation of antibodies, and in some cases, it can persist for up to six weeks [34]. In present study, the majority of the patients presented on the 5th and 7th day of illness, accounting for the low sensitivity of the IgM ELISA. The present study was comparable to Page J et al., and had 100% specificity and 100% PPV by HSV IgM ELISA [34].

The main factors determining Tzanck smear results are the stage of the infection at the time of sampling and the location of the lesion. Sensitivity varies according to the sites of infection and stages of lesions. The average duration of ulcerous skin lesions was about five days, and of ulcerous mucous membrane lesions about four days in men and six days in women. Older lesions have lower sensitivity in the Tzanck smear. The characteristic cytomorphological features of herpetic infection are obscured by the rapid loss of nuclear details in cells infected for a longer duration [35]. The longer average duration of ulcerous mucous membrane lesions is the cytomorphological explanation for the lower Tzanck smear sensitivity. In vesicular lesions, Tzanck smear specificity of 100% was obtained. Vesicles were more likely to yield a positive Tzanck smear (66.7%). Pustules showed positive Tzanck smears in 54.5%, while crusted-ulcer lesions had a low positivity rate of about 16%. This method has low sensitivity and does not distinguish between HSV-1 and HSV-2, nor between HSV and varicella-zoster virus infection [36].

In present study, 96% presented with ulcerous lesions, while the remaining 2% presented with vesicular lesions, which explains the low sensitivity of the Tzanck smear for HSV diagnosis. It is important to acknowledge that the present study showed Tzanck smear sensitivity at 10%, which was low compared to studies conducted by Oranje AP et al., and Ozcan A et al., [37,38]. However, it showed 100% specificity and PPV, which was comparable to Banihashemi M et al., [39].

The choice of the diagnostic assay depends on the purpose of the test. For the diagnosis of acute lesions, nucleic acid amplification assays are most appropriate due to their high sensitivity and specificity compared to other diagnostic methods for HSV. Therefore, PCR

can be considered a gold standard technique in HSV diagnostics. Laboratory confirmation of clinically suspected cases of herpes is necessary as it helps to identify the persons at risk of transmitting the infection. Molecular methods are a good alternative to cell culture.

Limitation(s)

In light microscopy, low sensitivity and specificity are attributed to the indistinguishable cytopathogenic effect specific to HSV. In the case of IgM ELISA, the appearance of antibodies after the onset of the disease varies, leading to low sensitivity.

CONCLUSION(S)

The PCR is a sensitive diagnostic method for HSV, but its use is restricted due to the cost and the requirement of trained technical staff. It is a rapid method for detection and is less vulnerable to interference by exposure to heat or substances that inhibit the growth of HSV. The greater rapidity and sensitivity need to be balanced against the greater reagent cost for PCR testing compared to virus culture.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jan 20, 2023
- Manual Googling: Jan 23, 2024
- iThenticate Software: Jan 27, 2024 (18%)

ETYMOLOGY: Author Origin

EMENDATIONS: 7

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. Yes

Date of Submission: **Jan 19, 2023**
Date of Peer Review: **Apr 18, 2023**
Date of Acceptance: **Jan 29, 2024**
Date of Publishing: **Jul 01, 2024**