

Isolation, characterization and acyclovir susceptibility of herpes simplex virus isolates among immunocompromised patients

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Abstract

Introduction: Herpes simplex virus (HSV) Type 2 primarily causes genital herpes, while HSV Type 1 is responsible for oral and facial lesions. The objective of this study was to isolate and characterize HSV from herpetic lesions among human immunodeficiency virus (HIV) infected patients and to evaluate their acyclovir susceptibility pattern. **Materials and Methods:** Blister fluid and swabs from ulcers were collected from patients with clinical diagnosis of HSV infection among patients attending the HIV clinic of two tertiary care centers – Medical College, Kolkata, and School of Tropical Medicine, Kolkata. These samples were cultured in the Vero cell line. Growth of virus was noted by observing the characteristic cytopathic effect of HSV, which was further confirmed by immunofluorescence and polymerase chain reaction (PCR). These isolates were then subjected to the Vero cells with serial dilutions of acyclovir for determining the susceptibility pattern. **Results:** Among the 52 samples received, 8 (15.38%) showed growth of HSV. After confirmation by immunofluorescence and PCR, all seven isolates from genital samples were identified as HSV-2 and the lone isolate from oral lesion was confirmed as HSV 1. Out of the eight isolates, 25% showed resistance to acyclovir. The overall isolation rate was more from genital blister than genital ulcer which was 46.15% and 2.86%, respectively. **Conclusion:** HSV was isolated in 15.38% of cases of clinical herpes. There was a higher isolation rate of virus from blister fluid as compared to ulcer scrapings. Acyclovir resistance in 25% of cases is alarmingly high.

Key words: Acyclovir resistance, culture, herpes simplex virus, polymerase chain reaction

INTRODUCTION

Herpes simplex virus (HSV) establishes latency in the sensory ganglia after initial acquisition, causing recurrent infections. HSV-2 infection is the primary cause of genital herpes and is one of the most prevalent sexually transmitted infections (STIs) worldwide. Genital herpes

is associated with substantial morbidity and neonatal herpes. HSV-1 infection is usually characterized by oral or facial lesions.^[1]

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The incubation period for genital HSV of either viral type ranges from 1 to 12 days. Many people experience marked signs and symptoms during primary infection, including bilateral lesions along with regional lymphadenopathy, headache, fever, malaise, and other symptoms. Primary infection may also be mild or entirely asymptomatic. Estimated 70%–90% of patients experience recurrences in the 1st year which are usually unilateral.^[2]

Coinfection between human immunodeficiency virus (HIV) and other STIs are common due to shared routes of sexual transmission. HIV-HSV-2 coinfection increases transmissibility of HIV in both sexes and progression to AIDS.^[3] HIV-HSV-2 coinfection has also caused genital ulcers of extensive and persistent nature, which reoccur frequently and show atypical clinical presentations.^[4] Severity of symptomatic HSV-2 has shown a correlation with low CD4 counts. In a study, it was seen that of 96 swabs taken from patients with CD4+ cell counts <50 × 10⁶/l, 56 (58.3%) were positive for HSV, compared with 27 of 127 (21.2%) swabs from patients with higher CD4+ cell counts ($P < 0.0001$).^[5]

This study was conducted to isolate HSV from the genital and oral herpetic lesions – ulcers and blisters, characterize it, and determine its susceptibility to acyclovir.

MATERIALS AND METHODS

Sample collection

After obtaining Institutional Ethical Clearance, this study was done among the patients attending the HIV clinic of two tertiary care centers – Medical College, Kolkata, and School of Tropical Medicine, Kolkata, during a period of 1 year (from April 2012 to March 2013).

Sample size calculation was done using the formula $4pq/d^2$, where P = prevalence, $q = 100 - p$, and d is allowable error. The prevalence of HSV infection was taken as 7%^[6] and the sample size was calculated as 52.

After obtaining informed consent, blister fluid/ulcer scrapings and swabs were collected from adult patients (18–55 years) with clinical diagnosis of oral or genital herpes. Their personal, demographical, and clinical data were obtained by a pretest questionnaire containing information on name, age, sex, socioeconomic status, occupation, marital status, contact history, medical history, sexual behavior, risk factors, knowledge of sexually transmitted disease (STD), and clinical symptoms. Patients below 18 years or above 55 years of age (they were

supposed to be not sexually active), those suffering from critical or deteriorating diseases, or those having history of receiving antiviral therapy except antiretroviral therapy (ART) were excluded from the study. The samples were processed at ICMR Virus Unit, Kolkata.

Culture

The slide prepared from scraping of ulcer was air-dried, fixed with alcohol, stained with Giemsa stain, and observed for the presence of multinucleate giant cells.

The blister fluid was collected in vial containing viral transport medium, vortexed, and filtered through a membrane filter (0.22 μm). Vero cell (ATCC, USA) monolayer (10⁵) was infected with the filtrate in a six-well plate containing the complete culture media. The culture was incubated at 37°C for 48–72 h with 5% CO₂. Most culture positives were obtained within 3 days under the standard incubation procedure though cultures were kept for 10 days.^[7] The plate was then examined under an inverted microscope for the evidence of characteristic cytopathic effects including cell lysis, syncytia, or multinucleate giant cell formation with altered nucleus of herpes virus infection. The supernatant was pipetted out and discarded. The cell layer was scrapped of, taken in a microcentrifuge tube, then vortexed, and centrifuged. The supernatant was used for DNA extraction and subsequent polymerase chain reaction (PCR).

Polymerase chain reaction

Nucleic acid extracted from the tissue culture supernatant fluid from the infected Vero cells showing characteristic cytopathogenic effect (CPE), using QIAmp Mini Elute Virus Spin Kit (Qiagen, Germany), was subjected to PCR. 20 μl of PCR mix was prepared by mixing 10 μl of 2X Enzyme Premix (25 mM MgCl₂, 2.5 mM each of dATP, dCTP, dGTP, dTTP, Taq DNA polymerase), 2 μl of 1:1 primer mix (30 mM each of upstream and downstream primers), 2 μl of total DNA in DNAase-free water, and 6 μl DNAase free water. The mixture was subjected to DNA amplification using the GeneAmp PCR System 9600 (Perkin Elmer Corp.). The primer was used to amplify HSV pol gene, 5' primer TGTGGTCGTCGACGATTGCAGCAT and 3' primer TGGGAGTGACCCGCGTGGTTCGA. The positive control in the PCR was a known culture-positive well-characterized HSV isolate while the negative control was DNAase-free water. Finally, PCR products (about 8 μl) mixed with bromophenol blue (2 μl) was loaded on 2% agarose gel for electrophoresis.

Immunofluorescence

Vero cell monolayer was infected with supernatant and incubated at 37°C for 24 h with 5% CO₂. The infected Vero cell monolayer was then washed twice with phosphate-buffered saline (PBS, pH 7.2) to remove the cell debris. The cells were then fixed with para-formaldehyde (4%) and blocked with 1% bovine serum albumin in 0.1% PBS-triton ×100 solution. The cells were again washed with PBS, and then, permeabilization was made with 0.1% triton X100 in PBS and incubated overnight with fluorescein isothiocyanate-labeled anti-HSV-1 and HSV-2 mouse monoclonal antibodies (DakoCytomation, Denmark). After washing with PBS, secondary rabbit polyclonal antibodies (DakoCytomation, Denmark) were added, and the cells were observed under epifluorescence microscope.

Acyclovir resistance detection

Isolates were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS). The viral isolates were inoculated on to the Vero cell monolayers growing in 96-well plates, containing EMEM with 2% FCS as diluent, 1% glutamine, and antimicrobials (penicillin, streptomycin, and amphotericin B). For every HSV isolate, eight serial 1:10 dilutions of acyclovir were added. Antiviral agents were omitted in the first well of every set to act as growth controls. Plates were then incubated at 37°C with 5% CO₂ for 72 h. After this incubation period, formation of CPE was observed using an inverted light microscope. Interpretation of the results was performed by comparing the titer obtained in the sets without antiviral agent with those obtained in the sets containing antiviral agent.^[8]

Analysis

The clinical features, laboratory parameters, and other data were entered in the Excel Spreadsheet (Microsoft Office, Redmond, Washington, USA). The statistical analysis of the clinical data was done using STATA version 13 (StataCorp LLC, Lakeway Drive College Station, Texas, USA). The data were summarized using mean along with standard deviation for continuous variables and frequency along with percentages for categorical variable. Chi-square test was used to check the categorical variables association, and $P < 0.05$ was considered statistically significant.

RESULTS

A total of 52 samples were received during the study period of 1 year (from April 2012 to March

2013). Among these patients, 30 (57.69%) were male and 22 (42.31%) were female. All patients included were between the ages of ≥ 18 years and ≤ 55 years. Among these patients, 44 were married, 2 were never married, one separated, and 5 widowed. There were 28 patients with single partner and 24 patients with multiple partners. There were 13 samples from the genital blister and 35 samples from the genital ulcer, which yielded a total growth in seven isolates. There were four oral samples of which one yielded growth [Table 1].

The growth of isolate was detected by immunofluorescence and PCR in all eight cases [Figure 1]. All the genital samples were confirmed as HSV-2 and the lone isolate of oral sample was HSV-1 [Figure 2]. Isolation was more from genital blister samples (46%) than genital ulcer samples (2.86%). Multinucleate giant cells were seen in scraping of four ulcer bases (three genital and one oral ulcer). Out of the eight isolates, 25% showed resistance to acyclovir. Table 2 shows the relationship between demographic characteristics and culture positivity.

DISCUSSION

Genital herpes is a common chronic STI worldwide with substantial morbidity caused mainly by HSV-2 and sometimes by HSV-1. The strongest known risk factor for the heterosexual transmission of HIV and other STIs is genital ulcer disease. Laboratory confirmation of genital herpes is important. The classical method for diagnosis and typing of HSV infections has been virus culture, while PCR is a modern technique.^[9] Viral culture has conventionally been regarded as the gold standard for diagnosis.^[10] However, viral isolation, HSV DNA detection by PCR, and HSV antigen detection by enzyme immunoassay or DIF, all are different methods used

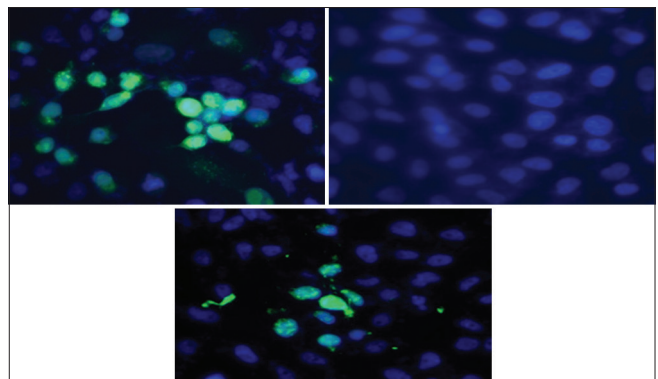


Figure 1: Shows confirmation of virus by immunofluorescence study. Virus control (herpes simplex virus-infected Vero cell layer). Cell control (noninfected Vero cell layer). Patient sample showing the presence of herpes simplex virus infection

Table 1: Virus isolation from clinical samples

Type of sample	Number of samples	Multinucleate giant cell detection	Number of isolates of HSV	Acyclovir resistance, n (%)
Genital blister	13	0	6 (46.15)	1
Genital ulcer	35	3	1 (2.86)	1
Total genital	48	3	7 (14.58) All HSV-2	2
Oral blister	1	0	1 (100)	0
Oral ulcer	3	1	0	0
Total oral	4	1	1 (25) All HSV-1	0
Total (genital + oral)	52	4	8 (15.38)	2

PCR and immunofluorescence was done only on culture positive isolates. PCR=Polymerase chain reaction; HSV=Herpes simplex virus

Table 2: The relationship between demographic characteristics and culture positivity

Features	Clinical herpes (n=52), n (%)	Culture positive (n=8), n (%)	P
Male	30 (57.69)	3 (37.5)	0.259
Single (unmarried/widow/separated)	8 (15.38)	1 (12.5)	1.000
Multiple partner	24 (46.140)	6 (75)	0.123

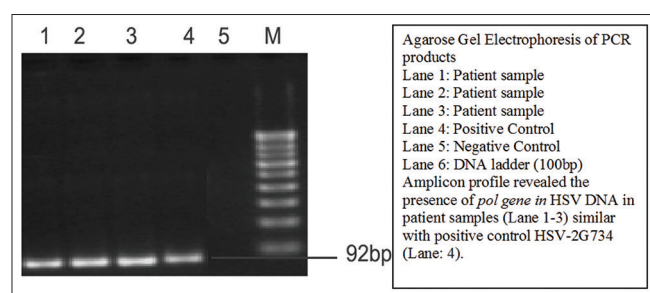


Figure 2: Polymerase chain reaction-based assay for typing herpes simplex virus. Extraction of viral DNA: using QIAmp® MiniElute® Virus Kit (QIAGEN). Primers: for *Pol* gene (IDT, India). Herpes simplex virus. Forward primer: 5'- CAG AAC TTC TAC AAC CCC CA -3'. Reverse primer: 5'- TAG ATG ATG CGC ATG GAG TA -3'

for direct virus detection.^[11] In this study, culture was done from the genital and oral ulcer and blister fluid. The culture-positive isolates were confirmed by indirect immunofluorescence and PCR.

In a study done in a STD clinic in Paris, it was found that among a total 278 cases of genital ulcer disease that were investigated, genital herpes accounted for 27% besides primary syphilis seen in 35% cases. Genital herpes was significantly associated with heterosexuality. Genital herpes was associated with HIV infection in the subgroup of MSWs.^[12] However, in a study done in India among 90 patients of genital ulcer disease, HSV ulcer was the most common. It was seen that sexual orientation was heterosexual (92.2%) or homosexual (2.2%).^[13] No such association could be found in this study as all 52 patients were heterosexual. The prevalence of HSV-2 was significantly higher in persons with multiple partners and in the reproductive age group.^[14] In this study, the inclusion criteria were patients in the

reproductive age group. However, no association was found between culture positive cases and multiple partners ($P = 0.123$). In this study, it was found that among the 52 samples comprising ulcer and blister fluid, 8 (15.38%) showed growth of HSV of which 7 were HSV 2 and one isolate of HSV 1. In a study done in Tanzania, out of 301 patients with genital ulcer, 192 (64%) had HSV 2 infection and 18 (6%) had HSV 1 infection.^[15] A study from India showed that among 90 patients of genital ulcer disease, 64 (71%) had HSV infection detected by PCR.^[13] HSV-1 and HSV-2 are the main cause of genital ulcers worldwide. Although HSV-2 is the major cause of genital lesions, HSV-1 accounts for half of new cases in developed countries.^[16] In this study, all the isolates from genital ulcer were identified as HSV-2 and the lone isolate from oral blister was identified as HSV-1.

Specimens obtained from vesicular lesions within the first 3 days after their appearance are the specimens of choice, but other lesion materials such as scrapings from older lesions or swabs of genital secretions should be obtained if suspicion of HSV infection is high.^[7] In this study, it was found that blister fluid had a better yield as compared to ulcer samples as shown by growth in 46.15% in the genital blisters and 2.86% in the genital ulcers. Among the oral samples, there was only one oral blister which showed growth while three oral ulcer samples did not show any growth.

The widespread use of acyclovir and the increasing number of immunocompromised patients have raised concern about an increase in acyclovir-resistant HSV. A study showed that there is rise in acyclovir

resistance in immunocompromised patients. A significant increase was observed, rising from 3.8% between 2002 and 2006 to 15.7% between 2007 and 2011.^[17] In the present study, it was seen that 25% of the isolates were resistant to acyclovir. In these resistant cases, topical cidofovir ointment was applied for the management and patients showed clinical improvement. Acyclovir is the antiviral treatment of choice but may lead to emergence of acyclovir-resistant HSV, due to mutations in the viral UL23 gene encoding for the acyclovir-targeted thymidine kinase protein.^[18]

Limitations

The limitations of this study were that direct PCR and immunofluorescence could not be done on the clinical samples.

CONCLUSION

Out of 52 samples, HSV was isolated from 8 (15.38%) samples. Isolation rate was more from blister samples (33.33% from both genital and oral blisters) than from ulcers (6.45% from genital ulcers). Emerging acyclovir resistance (25% of culture positive samples) is an alarming sign. A larger prospective study may be done to look for acyclovir resistance in clinical herpes cases.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

- Smith JS, Robinson NJ. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: A global review. *J Infect Dis* 2002;186 Suppl 1:S3-28.
- Feltner C, Grodensky C, Ebel C, Middleton JC, Harris RP, Ashok M, *et al.* Serological Screening for Genital Herpes: An Evidence Review for the U.S. Preventive Services Task Force. Rockville (MD): Agency for Healthcare Research and Quality (US); 2016. (U.S. Preventive Services Task Force Evidence Syntheses, formerly Systematic Evidence Reviews). Available from: <http://www.ncbi.nlm.nih.gov/books/NBK409117/>. [Last accessed on 2017 Mar 02].
- Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA, Hayes RJ. Herpes simplex virus 2 infection increases HIV acquisition in men and women: Systematic review and meta-analysis of longitudinal studies. *AIDS* 2006;20:73-83.
- Li D, Yang X, Zhang Z, Wang Z, Qi X, Ruan Y, *et al.* Incidence of co-infections of HIV, herpes simplex virus type 2 and syphilis in a large cohort of men who have Sex with men in Beijing, China. *PLoS One* 2016;11:e0147422.
- Bagdades EK, Pillay D, Squire SB, O'Neil C, Johnson MA, Griffiths PD. Relationship between herpes simplex virus ulceration and CD4+ cell counts in patients with HIV infection. *AIDS* 1992;6:1317-20.
- Schneider JA, Lakshmi V, Dandona R, Kumar GA, Sudha T, Dandona L. Population-based seroprevalence of HSV-2 and syphilis in Andhra Pradesh state of India. *BMC Infect Dis* 2010;10:59.
- Singh A, Preiksaitis J, Ferenczy A, Romanowski B. The laboratory diagnosis of herpes simplex virus infections. *Can J Infect Dis Med Microbiol* 2005;16:92-8.
- Cotarelo M, Catalán P, Sánchez-Carrillo C, Menasalvas A, Cercenado E, Tenorio A, *et al.* Cytopathic effect inhibition assay for determining the *in vitro* susceptibility of herpes simplex virus to antiviral agents. *J Antimicrob Chemother* 1999;44:705-8.
- Liu J, Yi Y, Chen W, Si S, Yin M, Jin H, *et al.* Development and evaluation of the quantitative real-time PCR assay in detection and typing of herpes simplex virus in swab specimens from patients with genital herpes. *Int J Clin Exp Med* 2015;8:18758-64.
- Marshall DS, Linfert DR, Draghi A, McCarter YS, Tsongalis GJ. Identification of herpes simplex virus genital infection: Comparison of a multiplex PCR assay and traditional viral isolation techniques. *Mod Pathol* 2001;14:152-6.
- Domeika M, Babayan K, Ismailov R, Shimanskaya I, Chudomirova K, Brilene T, *et al.* Survey of diagnostic services for genital herpes in fourteen countries in Eastern Europe. *Acta Derm Venereol* 2011;91:333-6.
- Hope-Rapp E, Anyfantakis V, Fouéré S, Bonhomme P, Louison JB, de Marsac TT, *et al.* Etiology of genital ulcer disease. A prospective study of 278 cases seen in an STD clinic in Paris. *Sex Transm Dis* 2010;37:153-8.
- Muralidhar S, Talwar R, Anil Kumar D, Kumar J, Bala M, Khan N, *et al.* Genital Ulcer Disease: How Worrisome Is It Today? A Status Report from New Delhi, India. *J Sex Transm Dis* 2013; Article ID 2013:203636.
- Nag S, Sarkar S, Chattopadhyay D, Bhattacharya S, Biswas R, SenGupta M. Seroprevalence of herpes simplex virus infection in HIV coinfecting individuals in Eastern India with risk factor analysis. *Adv Virol* 2015; Article ID 2015:537939.
- Nilsen A, Kasubi MJ, Mohn SC, Mwakagile D, Langeland N, Haarr L. Herpes simplex virus infection and genital ulcer disease among patients with sexually transmitted infections in Dar es Salaam, Tanzania. *Acta Derm Venereol* 2007;87:355-9.
- Glinšek Biškup U, Uršič T, Petrovec M. Laboratory diagnosis and epidemiology of herpes simplex 1 and 2 genital infections. *Acta Dermatovenol Alp Pannonica Adriat* 2015;24:31-5.
- Frobert E, Burrel S, Ducastelle-Lepretre S, Billaud G, Ader F, Casalegno JS, *et al.* Resistance of herpes simplex viruses to acyclovir: An update from a ten-year survey in France. *Antiviral Res* 2014;111:36-41.
- Mitterreiter JG, Titulaer MJ, van Nierop GP, van Kampen JJ, Aron GI, Osterhaus AD, *et al.* Prevalence of intrathecal acyclovir resistant virus in herpes simplex encephalitis patients. *PLoS One* 2016;11:e0155531.