

Development of a Western Blot Assay for Detection of Antibodies against HSV Using Purified HSV Virions Prepared by Sucrose Density Gradient

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Abstract

Objective(s)

Herpes simplex viruses (HSVs) have widespread and ubiquitous prevalence in the human population and they have received a great deal of attention due to the range of diseases, they caused as a result of an infection. It seems that the fast and reliable diagnostic methods are needed for detecting the herpes simplex virus type 1 (HSV1) antibodies especially in patients with HSV encephalitis, immunocompromised people, and neonatal infections. The aim of this study was designing a Western blotting method for HSV1 antibody detection, using the purified virus by sucrose density gradient centrifugation procedure.

Materials and Methods

The most reliable method for HSV detection is virus neutralization test but it needs cell culture preparation, high expertise, as well as the high amounts of serum samples. Considering the difficulties of this method, we tried to run a new one for HSV antibody detection by propagating the viruses and then purify them by sucrose density gradient centrifugation method. The purified viruses used as antigens in Western blotting assay.

Results

Diluted sera (1:100, and 1:200 dilutions) used in Western blotting and two-fold dilutions of the sera applied in virus neutralization test. Five of twenty seven samples were negative in Western blotting and the same results obtained in virus neutralization test. Comparing with our gold standard, the sensitivity and specificity of the developed assay were both 100%.

Conclusion

Our results show that the designed method is a reliable method for replacing the virus neutralization test in diagnostic laboratories. It can also, be used for confirming the ELISA results.

Keywords: Density gradient centrifugation, Herpes simplex virus type 1, Virus neutralization test, Western blotting

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Introduction

Herpes simplex virus (HSV) is an enveloped, double stranded DNA virus which is an important human pathogen (1-5). The virus infects mucosal epithelium or damaged cutaneous epithelium and causes many kinds of human diseases including cold sores, eye and genital infections, and encephalitis (6-8). HSV is one of the most widespread human viral infections. HSV becomes latent in the proximal ganglion following the initial infection, and can be reactivated for life (9, 10). The virus infection is the most common cause of sporadic, fatal encephalitis, and its incidence is approximately 40 to 50 cases per year (8, 10, 11).

Different methods are used for HSV antibody detection including Enzyme-linked immunosorbent assay (ELISA), Immunofluorescent (IF), and virus neutralization test (VNT), but virus neutralization tests are more narrowly specific than other serological tests such as ELISA or IF (12). VNT needs to be done in eukaryotic cell culture systems.

The HSV can grow in a variety of fibroblastic and epithelial cell types, causing a characteristic cell rounding or ballooning which develops within a few days and spreads rapidly. In combination with clinical presentation, the development of characteristic cytopathic effect (CPE) in culture is an instrument for diagnosis but it is necessary the CPE is confirmed by a variety of immunocytochemical or antigen capture tests. There are many difficulties to make cell culture as an expert, as well as, the virus is sensitive to dissociation and swabs should be kept in transport medium and cultured as soon as possible. PCR and real time PCR can detect the viral genome and sometimes (especially in encephalitis), virus isolation or its genome detection is difficult; therefore the other methods are required for HSV-1 diagnosis (13). In this regard, the serological techniques based upon antibody reactivity to HSV have been useful for assessment of diagnosis and the seroprevalence of infection. Some serological methods especially ELISA, have good sensitivity but their specificity is low; therefore their positive results should be

confirmed by a specific method. Western blotting method is a specific and reliable technique for confirming the ELISA positive results. For the present study, a novel method was used for detecting the HSV antibodies in human serum samples. In this method, the whole HSV-1 particles propagated and purified using sucrose density gradient centrifugation procedure. Then, the purified viruses used as antigen in Western blotting method for detecting the virus.

Materials and Methods

Virus and cell line

A wild type of herpes simplex virus isolated from labial vesicles of a patient and identified as HSV1, using monoclonal antibody. The virus propagated in Vero cell monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% heat inactivated newborn-calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C with an atmosphere of 5% CO₂ (14).

Virus preparation

Vero cells infected at a multiplicity of infection (moi) of 0.1 PFU/ cell, and viruses harvested from the growth medium when more than 75% of the cells showed the virus cytopathic effects (CPEs).

Virus titration by 50 percent tissue culture infectious dose (TCID₅₀) method

TCID₅₀ method used for determining the virus titer that required for VNT technique. Initially, Vero cell monolayer was prepared in a 48-well plate. One log serial dilutions (10⁻¹-10⁻⁶) of the virus made in serum-free DMEM. Each dilution used for infecting four wells of the plate and CPE results considered by comparing with positive (virus without any dilution) and negative control (just cells) wells (15, 16).

Purification of the virus by sucrose density gradient centrifugation

Vero cells infected at a multiplicity of infection (moi) of 0.1 PFU/ cell. After appearing the CPEs, the viruses harvested from the growth medium and purified on

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gradients of 30%, 40%, 50%, and 60% sucrose. Briefly, cell debris removed by centrifugation at 8000 g and 4 °C for 30 min and virus supernatant concentrated by precipitation at 100000 g for 2 hr from the clarified medium. The pellet re-suspended in TNE buffer, pH 7.5 (0.1 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA) and then purified on 30%- 40%- 50%- 60%- sucrose-TNE step gradient at 75000 g for 5 hr. Purified viruses collected, centrifuged at 80000 g and 4 °C for 2 hr then re-suspended in 500 µl TNE, stored at -80 °C and used as antigen in Western blotting.

Sample collection

Twenty seven serum samples collected from volunteers with or without history of herpetic disease. Three neonate serum samples which were HSV negative by ELISA, used as negative controls.

SDS-PAGE and Western blotting

Purified virus in sucrose density gradient centrifugation mixed with electrophoresis sample buffer (50 mM Tris pH 8.6, 10% glycerol, 2% W/V sodium dodecyl sulfate (SDS), and 0.1% bromophenol blue, (non reduced method)) and boiled for 10 min. sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) performed in 12% polyacrylamide and the separated proteins, then transferred to nitrocellulose paper. Bounded proteins on the nitrocellulose reacted with total HSV1 antibody, after blocking in 5% gelatin. All serum samples diluted by 1:100 and 1:200, then analyzed with Western blotting. Antibody against HSV1, detected by reacting the blots with alkaline phosphate- conjugated anti human antibody and visualizing by diaminobenzidine (DAB) as a substrate (17, 18).

Virus neutralization test (VNT)

Twenty seven serum samples collected from volunteers with or without history of herpetic disease and three negative sera tested for detection of neutralizing antibodies. Briefly, two fold dilutions of heat inactivated of each serum ranging from 1/2 to 1/256 prepared in

serum-free DMEM. Each dilution incubated with 100 TCID₅₀ of HSV1 for 1h at 37 °C, in a total volume of 200 µl. Duplicate 100µl samples added to Vero cells in 96-well micro titer plates. All inoculated cells including positive and negative controls added to DMED containing 2% heat inactivated newborn-calf serum and incubated at 37 °C. The neutralization titer of each serum considered as the reciprocal of the highest dilution, preventing CPE in the inoculated cells.

Results

Figure 1 shows Vero monolayer cells. These cells are established from the kidney of a normal adult African green monkey. They are adherent with fibroblastic appearance. The virus cytopathic effects appeared, twelve to twenty four hr after the cells were infected with HSV-1. HSV-1 CPE on Vero cells is shown in Figure 2. The important sign of CPE appearance in the cells includes ballooning of the infected cells and multinucleated giant cells formation.

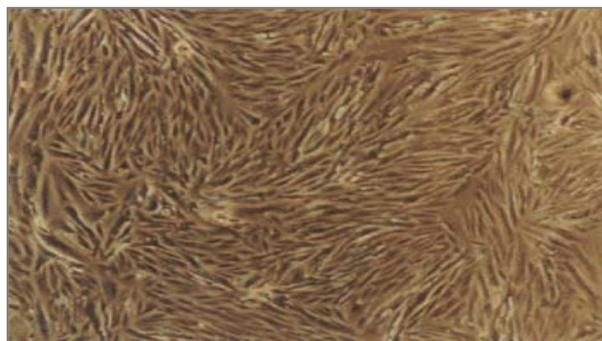


Figure 1. Vero monolayer cells. The cells are adherent with fibroblastic appearance.

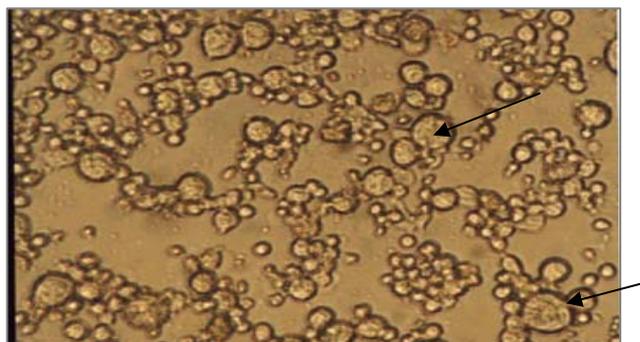


Figure 2. Cytopathic effects of HSV-1 on the Vero cells. The CPE includes ballooning of infected cells and the multinucleated giant cells formation (arrows).

CPE detected in infected cells and the ratio of the CPE positive wells to CPE negative

wells recorded (Table 1). Then, TCID₅₀ calculated as follows:

$$\text{Log TCID}_{50} = L - D (S - 0.5)$$

where, L= log of the lowest dilution, D= difference between the log of dilution, and S= the sum of proportions studied.

$$\text{Log TCID}_{50} = -1 - 1 (5.5 - 0.5) = -6$$

$$10^{-6} \text{ TCID}_{50} / 0.1 \text{ ml} = 10^{-1} \times 10^{-6} \text{ TCID}_{50} / \text{ml} = 10^{-7} \text{ TCID}_{50} / \text{ml}$$

Table 1. Results of virus titration (TCID₅₀ method) in Vero cells.

Virus dilutions	All wells	CPE positive wells	CPE positive wells/all wells
10 ⁻¹	4 wells	4 wells	4/4=1
10 ⁻²	4	4	4/4=1
10 ⁻³	4	4	4/4=1
10 ⁻⁴	4	4	4/4=1
10 ⁻⁵	4	4	4/4=1
10 ⁻⁶	4	2	2/4=0.5

Figure 3 shows the SDS-PAGE results for the purified viruses and a band with molecular weight of more than 116 KDa was observed.

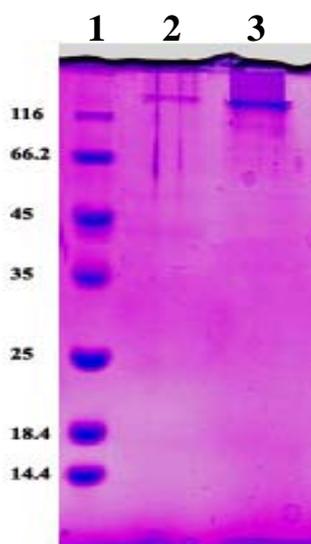


Figure 3. SDS-PAGE results for purified viruses using sucrose density gradient centrifugation. Number 1 is a protein size marker; number 2 is a negative control (cellular proteins without any viruses purified with the same protocol in sucrose density gradient centrifugation); and number 3 is purified viruses band.

The serum samples analyzed with Western blotting. Two dilutions of 1:100 and 1:200 of

the serum samples prepared and Western blotting done, using the purified-virus with sucrose density gradient. Our results showed that 22/27 of the 1:100 serum dilutions were positive and the negative control sera were negative. All positive sera and three negative sera were diluted to 1:200 and they used in the Western blotting method again. All of the 1:200 diluted-positive sera showed positive results in Western blotting method. Figure 4 shows the Western blotting results for 14 of positive sera and 3 negative control sera. For confirming the Western blotting results, all of the samples used in VNT and the same results obtained. Our results showed that both sensitivity and specificity of the designed assay to be 100%, in comparison with our gold standard assay.

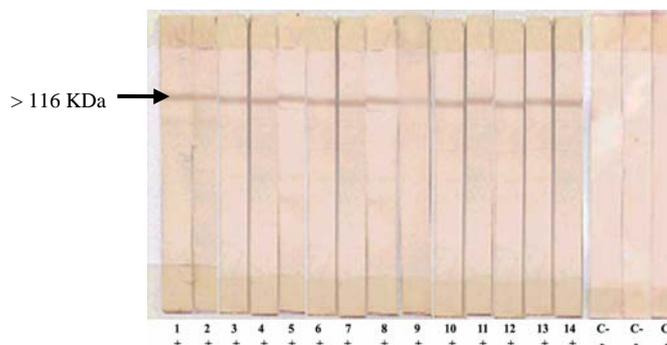


Figure 4. Western blotting results for 14 of positive sera and 3 negative control sera using purified HSV-1 as an antigen (1:200 dilutions of sera). The positive serum samples in the first Western blotting using 1:100 dilution of sera were diluted (1:200) again and used in Western blotting using purified HSV as an antigen. All positive sera in the first dilution were positive in the second Western blotting and the negative control sera showed negative results in the test.

Discussion

Herpes simplex virus type 1 (HSV1) remains a potentially serious health problem worldwide. All infected people, including asymptomatic ones, are potential sources for virus transmission. It causes a broad range of infections from asymptomatic ones to life threatening encephalitis. The virus is of more importance in infants and immunocompromised people. The diagnoses of herpes encephalitis and neonate infection present a special problem and rapid diagnosis is of importance because of the urgency of appropriate treatment (3, 6, 19, 20).

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HSV1 isolation or its genome detection is difficult especially in encephalitis; therefore a reliable serological method is necessary for the antibody detection. One of the serological diagnostic tests is VNT. The test is a reliable method for detecting the anti-HSV1 antibodies in serum samples, but it needs, cell culture preparation, high level of expertise, as well as high amounts of the sera.

Considering the difficulties of blood sampling especially in neonates and infants, the time required for VNT test and the need of facilities such as cell culture or an expert, we tried to run an alternative method. Western blotting was done using purified-virus with sucrose density gradient centrifugation as an antigen, two dilutions of 100 and 200 of the serum samples prepared and the assay carried out. Human neonate serum samples (which were HSV1 antibody negative in ELISA) used as negative controls. All the positive and negative samples confirmed by VNT.

Comparing with our gold standard, both sensitivity and specificity of the designed

Western blotting assay were 100%. Therefore, our data showed that the designed assay is a suitable method for detecting the HSV antibodies. This method does not need a large amount of serum and it is a fast and reliable one.

Conclusion

Our data showed that the designed Western blotting assay, using purified virus is a reliable method for HSV antibody detection. So, we recommend this method, to replace the VNT in diagnostic laboratories for the important cases such as HSV infections. Also, it can be used for confirming the positive results of ELISA.

Acknowledgment

We would like to thank Dr Seyed Ali Pourbakhsh (Avian Diseases Research and Diagnosis Dept., Razi Institute, Tehran, Iran) for kindly providing sucrose density gradient centrifugation facilities and protocols. The authors declare that they have no conflict of interests.

References

1. Cann AJ. DNA viruses. 1st ed. New York: Oxford University Press; 1999.
2. Dickerson FB, Boronow JJ, Stallings C, Origoni AE, Ruslanova I, Yolken RH. Association of serum antibodies to herpes simplex virus 1 with cognitive deficits in individuals with schizophrenia. *Arch Gen Psychiatry* 2003; 60:466-472.
3. Handler CG, Eisenberg Motamedifar M, Moattari A. Effects of sodium valproate on the replication of herpes simplex virus type 1: an in vitro study. *Iran J Med Sci* 2006; 3:28-32.
4. Shogan B, Kruse L, Mulamba GB, Hu A, Coen DM. Virucidal activity of a GT-Rich oligonucleotide against herpes simplex virus mediated by glycoprotein B. *J Virol* 2006; 80:4740-4747.
5. Handler CG, Eisenberg RJ, Cohen GH. Oligomeric structure of glycoproteins in herpes simplex virus type 1. *J Virol* 1996; 70:6067-6075.
6. Cohen GH, Dietzschold B, Ponce de Leon M, Long D, Golub E, Varrichio A, *et al.* Localization and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulates the production of neutralizing antibody. *J Virol* 1984; 49:102-108.
7. Hayashi K, Hooper LC, Chin MS, Nagineni CN, Detrick B, Hooks JJ. Herpes simplex virus 1 (HSV-1) DNA and immune complex (HSV-1-human IgG) elicit vigorous interleukin 6 release from infected corneal cells via Toll-like receptors. *J Gen Virol* 2006; 87:2161-2169.
8. Taylor TJ, Brockman MA, McNamee EE, Knipe DM. Herpes simplex virus. *Front Biosci* 2002; 7:D752-D764.
9. Higgins TJ, Herold KM, Arnold RL, Mc Elhiney SP, Shroff KE, Pachuk CJ. Plasmid DNA-expressed secreted and non-secreted forms of herpes simplex virus glycoprotein D₂ induced different types of immune responses. *J Infect Dis* 2000; 182:1311-1320.
10. Jenkins FJ, Turner SL. Herpes simplex virus: a tool for neuroscientists. *Front Biosci* 1996; 1:D241-D247.
11. Roizman B, Knipe DM. Herpes simplex viruses and their replication. In: Knipe DM, Howley PM. editors. *Fields virology*. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 2399-2509.
12. Mahy BWJ, Kangro HO. *Virology methods manual*. Great Britain, Glasgow: Academic Press; 1996.
13. Minson AC. Alphaherpesviruses: herpes simplex and varicellazoster. In: Topley & Wilson's microbiology and microbial infections. 9th ed. New York: Oxford University Press; 1998. p.325-339.
14. Morgan SJ, Darling DC. *Animal cell culture*, Bios Scientific Pub; 1993.
15. Mahy BWJ, Kangro HO. *Virology methods manual*. Oxford Washington DC: IRL Press; 1996.
16. Desselberger U. *Medical virology*. Oxford New York Tokyo: Oxford University Press; 1995.

17. Deutscher MP. Guide to protein purification. San Diego: Academic Press; 1990.
18. Sambrook J, Russell DW. Molecular cloning. 3rd ed. New York: CSHL Press; 2001.
19. Mattison HR, Eisenbeberg RJ, Reichman RC. Textbook of human virology. 2nd ed. St Louis: Belsh RB, Mosbey-Year Book; 1991.
20. Wagner EK, Bloom DB. Experimental investigation of herpes simplex virus latency. Clin Microbiol Rev 1997; 10:419-443.