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ELISA, InCell ELISA and Western Blot; alternative to the plaque reduction assay for *in vitro* determination of HSV susceptibility to acyclovir

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ABSTRACT • Background and Aims: Despite the fact that The Plaque Reduction Assay (PRA) has been proposed a standard method for *in vitro* determination of HSV susceptibility to acyclovir (ACV) by The National Committee for Clinical Laboratory Standards and a range of methods have been developed as tools for the determination of susceptibility of HSV to antiviral agents, there are several drawbacks associated with them, such as labor-intensive, time-consuming, high cost, and questionable reproducibility. The goal of this study was to test the usability of the ELISA, InCell ELISA and Western Blot (WB) techniques to improve capability for the determination of susceptibility of HSV-1 to antiviral agents. In addition to this aim of the study, we also investigated PancI and T98G cell lines in addition to Vero for determination of susceptibility of HSV-1. **Materials and Method:** For WB, extracted proteins from the cells infected with HSV-1 and treated with different concentration of ACV were used in SDS-PAGE and followed with immunoblot using HSV-1 specific antibody. Two ELISA methods were tested: in the first ELISA protein extracts obtained for the WB used to coat the 96 well high binding ELISA plates and standard ELISA protocol followed. In the second ELISA protocol that is also called InCell ELISA, cell growing in the 96 cell culture plates were treated for stabilization and permeabilization and then ELISA protocol followed. PRA, WB, and ELISAs were tested in Vero, PancI and T98G cell lines and compared for usability control cells. **Results:** This study showed that both WB and ELISAs detected HSV-1 presence in 24 hours after the virus infection which is 1 or 2 days earlier than the PRA result. WB result showed that it is more sensitive compared to PRA, such as PRA could not detected HSV-1 presence in 2 mg ACV, WB could detected HSV-1 survived in 2 mg ACV concentration. The study also showed that cytotoxic effects of the HSV-1 is faster in both PancI and T98G compared to the Vero cell line. **Conclusion:** The study showed that WB is found as the most sensitive method. Therefore, it may be more useful especially for determination of borderline resistant HSV-1 strains. Both ELISA tests are useful for *in vitro* determination of HSV susceptibility to ACV and more advantageous in terms of time and plating the susceptibility curve, and identification of IC50 value may be more accurate compared to that of PRA. We also noticed that cytotoxic effects of the HSV-1 is faster in both PancI and T98G compared to the Vero cell line.

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INTRODUCTION

Herpes virus (HSV 1-2) infections are one of the most common infectious diseases in humans. Especially patients receiving chemotherapy and hematopoietic stem cell transplants, and in immunocompromised patients, are frequently associated with severe and persistent infections with HSV-1 and antiviral resistance (1, 2). A number of *in vitro* assays are utilized for assessing the susceptibility of HSV to antiviral agents, including the plaque reduction assay (3), DNA hybridization assay (4), plating efficiency assay (5), plaque autoradiography assay (6), and PCR (7). Despite the fact that a range of methods have been developed as tools for the determination of susceptibility of HSV to antiviral agents, there are several drawbacks associated with them, such as labor-intensive, time-consuming, high cost, and questionable reproducibility. Currently the Plaque Reduction Assay (PRA) has been proposed a standard method for *in vitro* determination of HSV susceptibility to acyclovir by The National Committee for Clinical Laboratory Standards (NCCLS) (8). In addition to this, the PRA is the most commonly reported technique to which new methods are compared (9). However, PRA requires viral titration and prolonged incubation until the viral cytopathogenic effect is visible and is labor intensive, subjective and time consuming. Therefore, faster phenotypical assays using more sensitive and objective endpoints are preferable.

In this study, we aimed to develop faster, more sensitive and more convenient technique than the PRA. We aimed to investigate the presence of HSV-1 protein in infected cells. The protein presence is known to be directly related to the proliferation of the virus. Two related techniques, western blot (WB) and ELISA were tested for showing the protein presence and the results were compared to the results of the PRA. ELISA technique was performed in two ways: in the first ELISA extracted protein for the western blot used for coating the ELISA plates and followed ELISA protocol, in the second ELISA: ELISA protocol was followed on the cells growing on the ELISA plates after fixation and permeabilization of the cells. In addition to this aim of the study, we also investigated three different cells for determination of susceptibility of HSV-1. The results showed that WB and both ELISAs were sensitive enough to recognize HSV-1 presence. More importantly, the results of this study showed that both WB and ELISAs detected HSV-1 presence in 24 hours after the virus infection which is 1 or 2 days earlier than the PRA result. This study showed that WB is more sensitive compared to ELISA tests. Therefore, it may be more useful to determine the borderline resistant HSV-1. With these techniques (especially ELISA) plating the susceptibility curve, and identification of 50% inhibitory concentration (IC50) value may be easier. We also noticed that cytotoxic effects of the HSV-1 is faster in both PancI and T98G compared to the Vero cell lines.

MATERIAL and METHOD

Cells and viruse are used in the study

Vero, human gliobalstoma (T98G) and Pancreatic cancer (PancI) cell lines are used in the study. ACV susceptible standart HSV-1 (ATCC® VR-1493) was used to test the HSV-1 cytotoxicity and susceptibility to ACV. Cells were cultured in Dulbecco Minimal Essential Medium (DMEM) (Gibco, USA) supplemented with 10% FCS (Hyclone), and antibiotics (Penicillin-Streptomycin) (Gibco), and 1% L-Glutamin (Invitrogen Life Technologies), maintained at 37°C in a 5% CO_2/95% air atmosphere with change of medium every third day.

Plaque Reduction Assay

$1x10^5$ of Vero cells in DMEM containing 10% FCS 1% PS, 1% glutamine were inoculated in 24-well plates. HSV-1 (ATCC® VR-1493) was prepared as $1x10^4$/0,5 ml PFU in the DMEM containing 2% FCS and added on the 24 well plates in which

Vero cells grow. Six hours after HSV-1 infection mediums were discarded and washed with DMEM twice. ACV was diluted as 2 mg, 1 mg, 0,5 mg, 0.25 mg, 0.125 mg and 0,075 mg/ml in 2% FCS containing DMEM and added into the individual wells triplicate. Wells without HSV-1 and ACV were used as cell control. Cells infected with HSV-1 and not containing ACV were used as to follow the cytopathic effects of the HSV-1. PRA test were applied for PancI and T98G cells the same as described above.

Western blot

Twenty-fours after, the cell infected with HSV-1 and treated with different concentration of ACV and control cells were scrapped with the protein lysis buffer (50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF and 0.01 mg of aprotinin per ml). Protein extraction was followed with incubation of lysed cell in ice for 30 minutes and centrifuged at 10.000 g for 10 minutes. Supernatant were collected in clean epondorph tubes and protein concentrations were measured with BCA protocol (Pierce BCA Protein Assay Kit- Pierce Biotechnology Rockford, lL USA). Equalized amount of samples were denatured in loading buffer (0.25 M pH:6.8 tris, 40% glycerol, 8% SDS, 10% β mercaptoethanol) as 1.1 ratios at 95ºC for 4 minute. The cell-free extracts prepared from $1x10^5$ cells were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to Hybond ECL membranes (Amersham; Arlington Heights, IL, USA). Immunoblot protocol was followed using HSV-1 (Santa Cruz Biotechnology, sc-21719, USA) as primary antibody (monoclonal antibody developed against to Glycoprotein D (gD) which is a structural component of the herpes simplex virus (HSV) envelope and HRP conjugated secondary antibody (Pierce-31430- USA). Then the membrane was incubated with chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific- USA) and was exposed to x-ray film. For internal control determination, the same amount of the samples were run in SDS-PAGE and immunoblotted with the actin antibody (β-actin (Sigma-Aldrich, 2066- USA).

ELISA

Equalized amount of samples prepared for WB were mixed with the coating buffer ($NaCO_3$ 1,5 g, $NaHCO_3$ 2,93 g/lt, Ph 9,6) were placed into the High-bound 96 ELISA plates as triplicate and incubated at 4°C for 2-4 hours. Wells were washed with the washing solution (PBS+0,05% Tween-20) three times and blocked with blocking solution (PBS+1% BSA) at 37°C for 1 hour. After washing three times 1/100.000 diluted HSV-1 antibody (Santa Cruz Biotechnology, sc-21719) was added into the wells and kept at 37°C for 1 hour. The wells were again washed in PBS-T, and 100 µl of the appropriately diluted horseradish peroxidase (HRP) conjugated second antibody was added to each well. After 30 minutes of incubation with the second antibody at 37°C, the wells were again washed three times with PBS-T. Finally, 100 ul of a 1 mg/ml solution of o-Phenylenediamine dihydrochloride (OPD) (1 mg OPD, 0,5M citric acid, 0,05 M sodium phosphate (Na_3PO_4)+ 1 µl 30%H_2O_2) was added to each well and the absorbance (OD 570) of the wells was measured at various time intervals with a Biotek instrument (Synergy HT).

INCELL ELISA

Vero cells of $5x10^3$ in DMEM containing 10% FCS 1% PS, 1% glutamine were inoculated in 96-well plates. HSV-1 (ATCC VR-1493) was prepared as $1x10^4$/0,5 ml PFU in the DMEM containing 2% FCS and added on the 96 well plates in which vero cells grow. Six hours after HSV-1 infection mediums were discarded and washed with DMEM twice. ACV was diluted as 2 mg, 1 mg, 0,5 mg, 0.25 mg, 0.125 mg and 0,075 mg/ml in 2% FCS containing DMEM and added into the individual wells triplicate. Wells without HSV-1 and ACV were used as cell control. Wells infected with HSV-1 and not containing ACV were used as to follow the prolifi-

ration of the HSV-1. Twenty-four hours after, cells were fixed with 8% paraformaldehyde solution for 15 minutes and washed with washing solution 3 times. Later permeabilisation solution (0,25 ml 100XTriton-100+24,75 ml PBS) was added on cell and incubated for 30 minutes. After discarding permeabilisation solution, the cells were blocked with blocking solution (PBS+ 1% BSA) at 37°C for 1 hour. After washing, appropriately diluted HSV-1 (Santa Cruz Biotechnology, sc-21719) was added into the wells and kept at 37°C for 1 hour. The wells were again washed in PBS-T, and 100 µl of the appropriately diluted horseradish peroxidase (HRP) conjugated second antibody was added to each well. After 30 minutes of incubation with the second antibody at 37°C, the wells were again washed three times with PBS-T. Finally, 100 µl of a 1 mg/ml solution of o-Phenylenediamine dihydrochloride (OPD) (1 mg OPD, 0,5M citric acid, 0,05 M sodium phosphate (Na_3PO_4)+ 1µl 30%H_2O_2) was added to each well and the absorbance (OD 570) of the wells was measured at various time intervals with the Biotek instrument (Synergy HT).

RESULTS

Cytotoxic effects of the HSV-1 appeared earlier in the PancI and T98G cells compared to the Vero cell line.

The PRA test was performed in three cell lines as described in materials and methods section. While the cytotoxic effect of the HSV-1 appeared between 60 to 72 hours in the Vero, it appeared between 44 to 54 hours in the PancI and T98G cell lines (Table 1).

Table 1 Time for cytopathic effect appaerance of HSV-1 in different cells

Cell	hours
Vero	60-72
T98G	44-54
Panc I	44-54

The cytotoxic effect of HSV-1 has emerged in the 1 mg dose of ACV in the PRA

At 2 mg of ACV concentration, no cytotoxic effect appeared. The first seen cytotoxic effect has emerged in the 1 mg concentration. When Vero cell used as recommended by the NCCLS, the cytotoxic effect appeared at the earliest 60 hours depending on the amount of ACV used in the cell infected by the HSV-1. At the higher doses of ACV, which cause less HSV-1 proliferating in the cell, the observed cytotoxic effect was prolonged up to 72 hours.

ELISAs can detect HSV-1 at 24 hours

ELISA performed with the protein extract prepared for the WB detected HSV-1 presence at 24 hours after ACV addition of the HSV-1 infected cells. While there were no HSV-1 at the 2 mg ACV, ELISA detected HSV-1 presence at the 1 mg and lower concentration of ACV (Figure 1 and 2). In-Cell ELISA performed as described in the material and method section resulted in likewise the ELISA (data not shown). For the ELISA result, in simple linear regression analysis, r^2 value was found as 0,9768 (Figure 3).

Western Blot is more sensitive for detection of HSV-1 presence

WB was performed at 24th hour with the proteins extracted from the cells in which HSV-1 infected and treated with the different concentration of ACV and control cells. It showed more sensitive results compared to PRA and ELISAs' reults. Although both ELISAs and PRA could not detect HSV-1 presence in 2 mg ACV, WB could detect HSV-1 survivied in 2 mg ACV concentration (Figure 4). In WB, HSV-1 presence which is inversely related with the ACV amount increased signal dramatically when ACV concentration was lower than 0.25 mg.

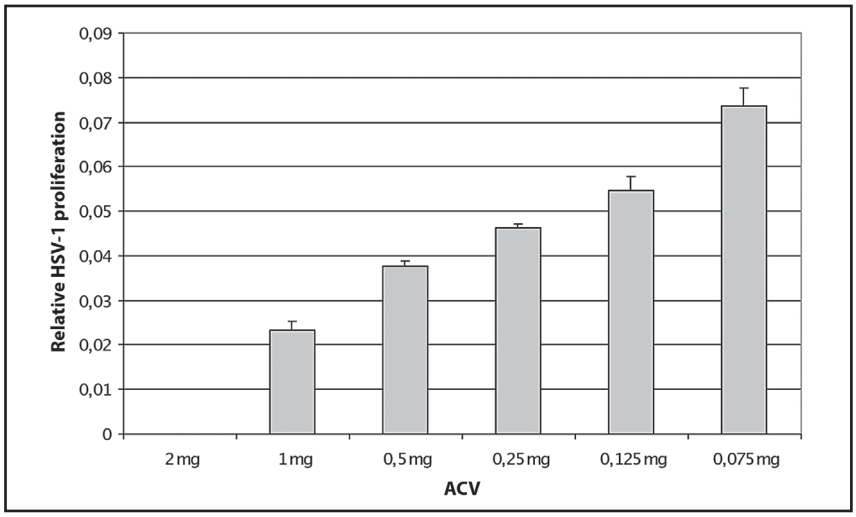

Figure 1 Vero cells in wells were infected with HSV-1 and treated with the different concentration of ACV. Twenty-four hours later, proteins were extracted, and ELISA protocol was followed as described in the material and method section. Results were graphed.

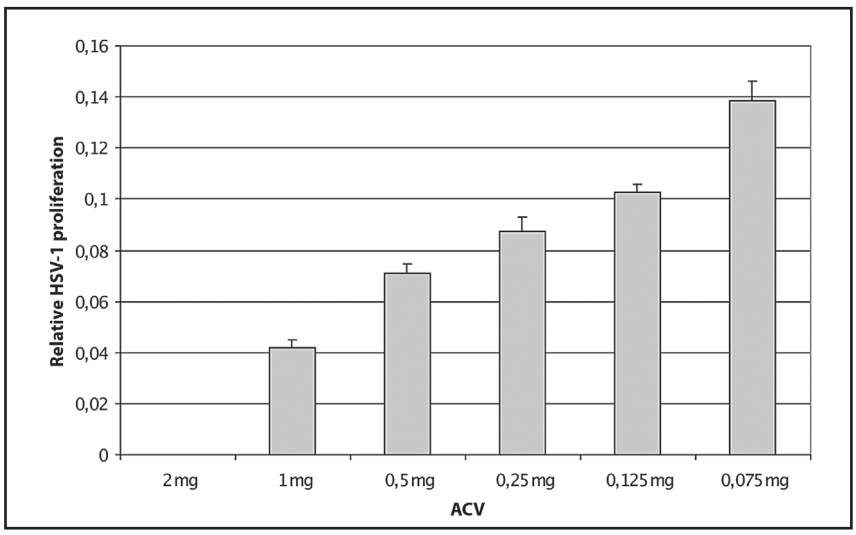

Figure 2 T98G cells in wells were infected with HSV-1 and treated with the different concentration of ACV. Twenty-four hours later, proteins were extracted, and ELISA protocol was followed as described in the material and method section. Results were graphed.

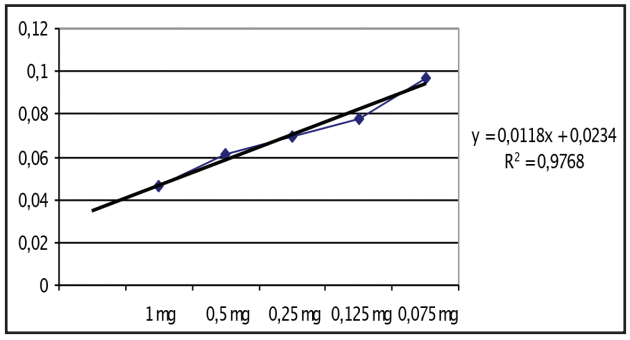

Figure 3 Linear regression analysis of T98G ELISA test results.

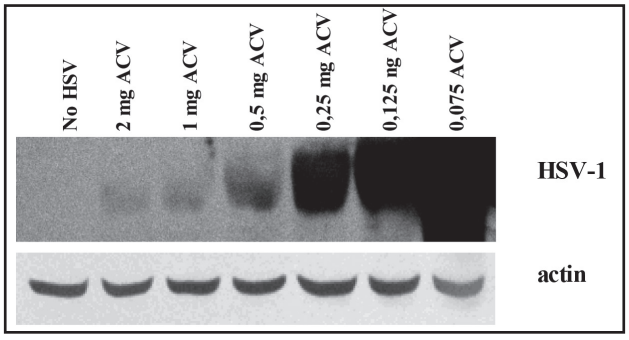

Figure 4 T98G cells in wells were infected with HSV-1 and treated with the different concentration of ACV. Twenty-four hours later, proteins were extracted, and WB protocol was followed as described in the material and method section.

DISCUSSION

Antiviral susceptibility testing of HSV clinical isolates is important because of the frequency of infection of immunocompromised hosts with resistant HSV strains and the increasing worldwide use of antiviral analog therapy. A number of *in vitro* assays are utilized for assessing the susceptibility of HSV to antiviral agents, including the PRA (3), DNA hybridization assay (4) plating efficiency assay (5), plaque autoradiography assay (6), and PCR (7). Although, plaque reduction assay (PRA) has been proposed a standard method for *in vitro* determination of HSV susceptibility to ACV by The NCCLS, PRA requires viral titration and prolonged incubation until viral cytopathogenic effect is visible and is labor intensive, subjective and time-consuming. Faster phenotypical assays using more sensitive and objective endpoints are preferable. WB is a strong technique for detection for protein. Since we did not find any study related with a drug susceptibility test using WB in the literature, we aimed to explore the usability of WB for the HSV-1 susceptibility to ACV. Results of WB showed that WB is more advantageous than the PRA in terms of sensitivity and time. In PRA, the cytopathic effect of HSV-1 appeared between 60 to 72 hours depending on the HSV-1 amount in the cell whereas WB detected HSV-1 presence at 24 hours after the infection. More importantly while PRA could detect HSV-1 presence at the 1 mg and lower concentration of ACV, WB could detect HSV-1 at the concentration of 2 mg of ACV. To date, the PRA remains the only assay shown to correlate laboratory-determined IC50 values with a clinical disease outcome of the patient (3, 10). However, even the PRA, which is robust for distinguishing thymidine kinase-negative HSV, may limit accurate identification of borderline-resistant, such as some TK-altered or DNA Pol-altered viruses if standard controls are not consistently utilized. Therefore, WB might be useful to resolve this issue since it is more sensitive compared to the PRA. It would be expected that WB can detect borderline-resistant HSV-1 strain more efficiently than the PRA.

In addition to WB, we also investigated ELISA test usability for the HSV-1 susceptibility to ACV. InCell ELISA was tested for assessing the susceptibility of HSV to antiviral agents and found results similar to our result in 1987 by Rabalais et al.(11). InCell ELISA was standardized and named as Microplate ELISA by Leahy et al. in 1994 (12). Later, InCell ELISA was tested in 1996 Safrin et. al. for assessing the susceptibility of clinically obtained HSV-1s to antiviral agents (13). After the study accomplished by Safrin et al. InCell ELISA was not considered or used by any other researcher for the antiviral susceptibility testing of HSV. Interestingly the other ELISA protocol used in this study was not tested previously by any study with our knowledge. The results of both ELISAs tested in this study showed that they are advantages over PRA: first both ELISAs give result faster than PRA, secondly plating the susceptibility curve and identification of IC50 value is easier compared to the PRA. In simple linear regression analysis, the r^2 value was found as 0,9768 which shows a very good correlation between HSV-1 presence and ACV concentration. Therefore finding IC50 value from the equation would be more accurate. We think that both ELISA techniques and WB will be more valuable for assessing the susceptibility of HSV to antiviral agents and may replace PRA after studying more HSV-1 from clinical samples including ACV resistant and borderline resistant strains. Another important finding of this study is the advantages of the usage of the different cell lines instead of Vero. It is known that HSV-1 has the high ability to infect the different cells. Therefore, we tested PancI and T98G cells for HSV-1 infectivity. Results of this study showed that PRA give a faster result in PancI and Vero cells compared to the Vero cell. The study also showed that both pancI and T98G cell can be used in the WB and ELISAs protocols.

In summary, in this study we tested ELISA, InCell

ELISA and WB for *in vitro* determination of HSV susceptibility to ACV. Results of the study showed that WB is the most sensitive method therefore it may be more useful especially for determination of borderline resistant HSV-1 strains. Both ELISA tests are more advantageous in terms of time and the better determination of IC50 values compared to that of PRA.

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