

Evaluation of an in - house ELISA for detecting herpes simplex virus antigen in comparison to conventional cell culture, shell vial cell culture and a commercial ELISA kit

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Bhattarakosol P, Punnarugsa V, Mung-Mee V. Evaluation of an in - house ELISA for detecting herpes simplex virus antigen in comparison to conventional cell culture, shell vial cell culture and a commercial ELISA kit. Chula Med J 2001 Jan; 45(1): 11 - 9

- Objective** : *To evaluate an in - house ELISA for detecting herpes simplex virus antigen (HSV-antigen).*
- Design** : *Experimental design*
- Setting** : *Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.*
- Subjects** : *118 clinical specimens collected from female patients with herpes genitalia (Bangrak Hospital)*
- Methods** : *All specimens were assayed for the presence of HSV-antigen by conventional cell culture (CCC) , shell vial cell culture (SVC) , an in - house ELISA (ELISA-P) and a commercial ELISA kit (ELISA-A).*
- Results** : *SVC was the most sensitive method, detecting 62.71 % of all samples, followed by CCC (41.53 %), ELISA-P (31.36 %) and ELISA-A (18.64 %).*

Conclusion : *In house ELISA (ELISA-P) was insufficiently sensitive for HSV-antigen detection when compared to the SVC viral isolation method; but it was superior to the commercial kit (ELISA-A). The SVC viral isolation method, is the most convenient and sensitive method for laboratory diagnosis of herpes simplex virus infection.*

Key words : *HSV-antigen, SVC, CCC, ELISA.*

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Received for publication. September 10, 2000.

ภาวพันธ์ ภัทรโกศล, วรณา พรณรรักษา, วนิตา มั่งมี. การประเมินผลวิธีอินเฮาส์อีไลซ่าเพื่อตรวจหาเฮอริปัสซิมเพล็กซ์ไวรัสแอนติเจนเปรียบเทียบกับวิธีคอนเวนชันนอลเซลล์คัลเจอร์ เซลล์ไวอัลเซลล์คัลเจอร์ และชุดน้ำยาสำเร็จรูปอีไลซ่าจากบริษัท. จุฬาลงกรณ์เวชสาร 2544 ม.ค; 45(1): 11 - 9

- วัตถุประสงค์** : เพื่อประเมินวิธีอินเฮาส์อีไลซ่าเพื่อตรวจหาเฮอริปัสซิมเพล็กซ์ไวรัสแอนติเจน
- รูปแบบการวิจัย** : การศึกษาแบบ experimental
- สถานที่ทำการศึกษา** : ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ ๙ 10330
- ประชากรที่ศึกษา** : ตัวอย่างสิ่งส่งตรวจจำนวน 118 ตัวอย่างเก็บจากผู้ป่วยหญิงที่มีอาการแสดงของโรคเริ่มที่อวัยวะสืบพันธุ์ที่โรงพยาบาลบางรัก กรุงเทพฯ ๙
- วิธีการศึกษา-วัดผล** : ทำการตรวจวิเคราะห์หาแอนติเจนของเฮอริปัสซิมเพล็กซ์ไวรัสจากตัวอย่างทั้งหมดด้วยวิธีคอนเวนชันนอลเซลล์คัลเจอร์ เซลล์ไวอัลเซลล์คัลเจอร์ วิธีอินเฮาส์อีไลซ่า และชุดน้ำยาสำเร็จรูปอีไลซ่าจากบริษัท
- ผลการศึกษา** : เซลล์ไวอัลเซลล์คัลเจอร์เป็นวิธีที่มีความไวที่สุด เพราะสามารถตรวจพบแอนติเจนของเฮอริปัสซิมเพล็กซ์ไวรัสได้ร้อยละ 62.71 จากตัวอย่างทั้งหมดตามด้วยวิธีคอนเวนชันนอลเซลล์คัลเจอร์ร้อยละ 41.53 วิธีอินเฮาส์อีไลซ่าร้อยละ 31.36 และชุดน้ำยาสำเร็จรูปอีไลซ่าจากบริษัทร้อยละ 18.64 ตามลำดับ
- วิจารณ์และสรุปผล** : วิธีอินเฮาส์อีไลซ่ายังไม่เหมาะสมในการตรวจหาแอนติเจนของเฮอริปัสซิมเพล็กซ์ไวรัสเมื่อเปรียบเทียบกับวิธีเซลล์ไวอัลเซลล์คัลเจอร์ซึ่งเป็นวิธีเพาะแยกเชื้อ แต่วิธีนี้ยังให้ผลดีกว่าการตรวจด้วยชุดน้ำยาสำเร็จรูปอีไลซ่าจากบริษัท วิธีเซลล์ไวอัลเซลล์คัลเจอร์ซึ่งเป็นวิธีเพาะแยกเชื้อยังเป็นวิธีที่สะดวกที่สุด และมีความไวมากที่สุดในการตรวจวินิจฉัยโรคติดเชื้อเฮอริปัสซิมเพล็กซ์ไวรัส

Herpes simplex virus (HSV) is a common cause of genital lesions and known to be a sexually transmitted disease. However, there are a number of infectious agents that can also cause genital ulcers. Therefore, a rapid and sensitive method for detecting HSV is necessary to help in improvement of patient care.

Laboratory diagnosis for HSV is composed of isolation, antigen detection and antibody detection. Among these, isolation of virus is accepted to be the gold standard method but the main disadvantage of isolation is that it is time consuming. Conventional cell culture (CCC) takes 5-7 days to observe the presence of cytopathic effect (CPE) on cell culture.^(1,2) The shell vial cell culture (SVC) has been developed and has an advantage because it is performed with centrifugation and takes only 2-3 days.⁽³⁻⁵⁾ For antigen detection, a commercial enzyme linked immunosorbent assay (ELISA) kit has been developed and widely used, since it is rapid and easy to perform.⁽⁶⁻¹⁰⁾ In the present study, an attempt was made to develop an in-house ELISA system and the performance of the test was compared to CCC, SVC, and a commercial ELISA kit (Enzygnostic, Beringwerk, Germany).

Materials and Methods

Patients and clinical specimens The study population was women who visited the Venereal Disease Clinic of the Department of Communicable Diseases Control, Bangrak Hospital, Bangkok, Thailand. A total of 118 samples was collected from patients with the clinical appearance of herpes genitalia as diagnosed by a physician. Different techniques were used to collect the specimens at various stages of disease according to the nature of

the lesion. Vesicle fluid was collected in vesicle stage. The ulcer and crust were collected by scraping with cotton swabs. All clinical specimens were placed in cold viral transport media, kept at 2-8 °C and then transferred to Viral Laboratory Unit, Department of Microbiology, Chulalongkorn Hospital, Chulalongkorn University, Bangkok, Thailand within 6 hours and kept at -70 °C until used.

The specimens were freeze-thawed once then centrifuged at 4 °C, 2500 rpm for 10 min. The supernatant was collected and used immediately as the inoculum for viral isolation. The remainder of the specimen was stored at -70 °C for ELISA.

Conventional cell culture (CCC) Approximately 1.5×10^5 Vero cells per ml resuspended in Growth medium (M199 supplemented with 10 % fetal bovine serum and antibiotics) were grown for 2 days in a culture tube. The amount of 0.2 ml of each specimen was inoculated and adsorbed for one hour. Subsequently, the specimen was discarded and the culture was maintained in maintenance medium (M199 with 2 % of fetal bovine serum and antibiotics). The viral CPE (enlarged round cells and multinucleated cells) was observed daily until 7 days. The experiments were performed in duplicate and standard HSV-1 strain KOS and HSV-2 strain LB were run in parallel as controls.

Shell vial cell culture (SCV) Vials (16 X 50 nm) containing a 12 nm diameter round coverslip seeded with Vero cells were inoculated with 0.2 ml of clinical specimens. Each of the specimens was assayed in duplicate. Vials were centrifuged at 2,500 rpm at 26 °C for one hour. Thereafter, the inoculum was discarded and replaced with maintenance medium. After incubating at 37 °C for 18 hours, the coverslips

were washed three times with phosphate buffered saline (PBS), pH 7.4, and air-dried. They were then fixed in cold acetone at -20°C for 10 min, air-dried and stained for the presence of HSV infected cells by an indirect immunofluorescence assay.

Indirect immunofluorescence assay After the fixing process, the cells were covered with 0.1 ml of 1: 20 dilution of polyclonal rabbit anti-HSV-2 antibody (Dakopatts, Denmark) for 30 min in moist chamber at 37°C . The cells were then washed with PBS 3 times for 5 min, followed by the addition of 0.1 ml of a 1: 40 dilution of swine anti-rabbit immunoglobulin conjugated with FITC. After incubating another 30 min, the cells were washed again 3 times with PBS. Finally, the cells were counter stained with Evan's blue 1: 30000 for 5 min, rapidly rinsed with distilled water and mounted with glycerol-PBS buffer. The cells were then ready for examination using Fluorescent Microscopy.

ELISA-A The Enzygnostic kit (Beringwerk, Germany) used for detecting HSV antigens in clinical specimens, is based on a double sandwich principle. The HSV antigen in clinical samples will bind to the HSV specific antibodies which are coated on the well and then other monoclonal antibodies that recognize both HSV-1 and HSV-2 conjugated with horseradish peroxidase are added. The specific immune complexes are detected by enzymatic reaction after adding TMB substrate. A positive sample is determined when the optical density (OD) was greater than 2 times of the OD from control reagent wells.

In - house ELISA (ELISA-P) The system was developed based on a double sandwich principle similar to ELISA-A. The coating antibody was rabbit anti-HSV-2 antibody (Dakopatts, Denmark) and the

second antibody for detecting HSV antigens was rabbit anti-HSV-2 antibody conjugated with horseradish peroxidase (Dakopatts, Denmark). Checkerboard titration was done according to the methods described by Voller et al.⁽¹¹⁾

The ELISA-P was developed in 96-well Microtiter plate (Nunc Immuno Maxisorp F16, Denmark). The wells were coated with 100 μl of rabbit anti-HSV-2 antibody (1:800) in carbonate buffer, pH 9.6 and incubated at 37°C . After 2 hours incubation, the wells were washed 5 times with PBS and 150 μl of blocking buffer [2 % bovine serum albumin fraction V, (Sigma, USA) in PBS] was added to each well and the plate was kept at 4°C for another 2 hours. After washing with PBS containing 0.05 % Tween 20 (Sigma) (PBS-T), 100 μl of the diluted clinical sample (1:2) was added. The reaction was kept for 16 hours or overnight at 4°C . On the next day, the plate was washed and then 100 μl of rabbit anti-HSV-2 conjugated with horseradish peroxidase (1: 1000) was applied and incubated at 37°C for one and a half hour. The bound antigen-antibody complexes were detected by adding 100 μl per well of 0.67 % 1,2-phenylenediamine dihydrochloride (Dakopatts, Denmark) for 20 min at room temperature. The reaction was stopped by 100 μl per well of 4 N H_2SO_4 . The optical density (OD) at 492 nm was determined. Control wells for each specimen were run at the same time in a separate well coated with normal rabbit serum to detect any nonspecific reaction present in the rabbit serum. The OD of the positive sample was calculated as an OD difference between the reacted anti-HSV coated well and the normal rabbit serum coated well of greater or equal to 0.1.

Results

A total of 118 specimens collected from suspected herpes lesions with different stages of lesion were simply classified into 2 groups; vesicular lesion and ulcer-crust lesion. There were 38 samples from vesicular lesion and 80 from ulcer-crust lesion. They were assayed for the presence of HSV by means of cell culture techniques i.e., CCC and SCV for viral isolation and the two ELISA techniques for detecting HSV antigens. The results are shown in Table 1. Comparing the results between two viral isolation methods, SCV detected 74 out of 118 (62.71 %) samples while CCC detected 49 (41.53 %) samples. Among those 49 samples positive by CCC, 33 (67.34 %) samples showed positive CPE after one day of incubation, 12 (24.49 %) positive samples were found on the second day, 1 (2.04 %) sample on the third day and another 3 (6.12 %) samples on the fourth day. For HSV antigen detection using ELISA, the in-house ELISA (ELISA-P) detected more positive samples than the commercial ELISA kit (ELISA-A); 37 (31.36 %) vs 22 (18.64 %), respectively. All positive samples from ELISA-A (22 samples) were positive according to

the result of ELISA-P. The 37 positive samples from ELISA-P were all detected positive by SCV, but 2 out of these were negative by CCC. One sample with positive ELISA-A result was negative isolation by CCC. The percentage of positive specimens were high in specimens collected from vesicular lesions in all assay types (Table 1). Evaluation of the tests including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) as well as accuracy were calculated and are shown in Table 2. The sensitivity varied depending on the assay method ranging from 29.73 %-71.43 %. The specificity was between 97.10 % to 100 %. The PPV was ranged from 94.59 % -100 % whereas NPV ranged from 45.83 % -100 %. According to the results, ELISA-P was superior in the detection of HSV antigen compared to ELISA-A, 71.43 % (35/49) vs 42.86 % (21/49) and CCC, detected 50 % (37/74) vs 29.73 % (22/74) compared to SCV (Table 1 and 2). Not only the sensitivity and specificity but also the accuracy of ELISA-P was better than that of ELISA-A i.e., 86.44 % vs 75.42 % and 68.64 % vs 55.93 %, respectively (Table 2).

Table 1. HSV positive samples determined by CCC, SCV, ELISA-A, and ELISA-P.

Methodology	Vesicular lesion n = 38 (% positive)	Ulcer-crust lesion n = 80 (% positive)	Total samples n = 118 (% positive)
CCC	21 (55.26)	28 (35)	49 (41.53)
SCV	27 (71.05)	47 (58.75)	74 (62.71)
ELISA-A	12 (31.58)	10 (12.5)	22 (18.64)
ELISA-P	15 (39.47)	22 (27.5)	37 (31.36)

Table 2. Evaluation of the tests by comparison between assays.

Methods	% Sensitivity	% Specificity	% PPV	% NPV	% Accuracy
ELISA-P/CCC	71.43	97.10	94.59	82.72	86.44
ELISA-A/CCC	42.86	98.55	95.45	70.83	75.42
ELISA-P/SCV	50	100	100	54.32	68.64
ELISA-A/SCV	29.73	100	100	45.83	55.93
ELISA-A/ELISA-P	59.46	100	100	100	59.46
CCC-SVC	66.22	100	100	63.77	78.81

Discussion

Among the several methods used in Viral laboratory units for the diagnosis of HSV infection, detection of HSV by viral isolation using cell culture technique is considered the best because it detects the presence of infectious HSV virions in clinical specimens. It is the "gold standard" with which all other assay systems are compared for HSV detection, although it is time consuming. In this present study, we tried to develop an in - house ELISA (ELISA-P) and evaluate our system compared to a commercial kit (ELISA-A) that uses the same principle. Since viral isolation using cell culture is the gold standard of diagnosis of HSV infection, CCC and SCV were included.

Our in - house ELISA (ELISA-P) was developed based on the double sandwich principle using a polyclonal antibody specific to HSV-2 not HSV-1 because Yoosook and her colleagues⁽⁸⁾ reported that 98.4 % of genital herpes in Thai patients were infected by HSV-2. Although there are very few cases caused by HSV-1 infection, the polyclonal antibody used in this system can also detect HSV-1 because both HSV-1 and HSV-2 have a 50 % antigenic similarity.⁽¹²⁾ Our results showed SVC was more sensitive than CCC (62.71 % vs

41.53 %, Table 1) corresponding to many previous reports.⁽³⁻⁵⁾ This difference might be because the staining of virus - infected cells in SVC by immunofluorescent assay enhanced the positive results while CCC was detected by CPE visualization. The ELISA-P system detected only half (50 %) of the SVC positive samples whereas commercial ELISA-A was much less sensitive (29.73 %). Although the sensitivity of ELISA tests was very low, the specificity was 100 % and the accuracy of ELISA-P was greater than that of ELISA-A. (Table 2). Considering types of specimen, the SVC method detected 71.05 % (27/38) of vesicular lesions and 58.75 % (47/80) of ulcer-crust lesions (Table 1). We previously demonstrated that SVC is the most sensitive method for detecting infectious virions and the culture yield is highest when the specimen is taken from vesiculopustular lesion.⁽¹³⁾ Other methods, such as CCC, ELISA-P and ELISA-A showed similar results. The sensitivity of the tests vary depending on many factors, for example type of specimen, transport system, principle of the test, time of incubation, etc. In this work, the time of the specimen reaction with antibody was different; ELISA-A was only 2 hours (recommended by the manufacturer) but ELISA-P was overnight (16 hours). Thus, the incubating time might

effect the sensitivity of the test. According to this study, viral isolation was shown to be more sensitive than antigen detection. It is probably because the viral isolation requires only small number of infectious virions in clinical specimens. The advantage of HSV antigen detection by ELISA has been claimed to be that it can detect efficiently all viral antigens, not only infectious virions. Thus, the antigen detection can be useful even in the late stage of the lesion where no infectious virions are produced and viral isolation will result in a negative result. However, we could not demonstrate that advantage in either vesicular lesions or ulcer-crust lesions.

In conclusion, we hereby demonstrate that SVC is still the best method for HSV detection and specimen collection from vesicular lesions increases the sensitivity of detection. At present, many commercial kits for HSV antigen detection using different principles have been developed such as biotin-streptavidin ELISA,⁽¹⁰⁾ enzyme linked fluorescent immunoassay⁽¹⁴⁾ and membrane enzyme immunoassay.⁽¹⁵⁾ Using different principles, the sensitivity of HSV antigen detection may be increased; but unfortunately we did not include these in this study. HSV is easy to culture from lesions but in other herpetic disease such as herpes simplex encephalitis the sensitivity of virus isolation is low. This is probably due to the low amount of virions in the cerebrospinal fluid. Thus, detection of HSV nucleic acid using polymerase chain reaction (PCR) has been developed and it will be helpful in early diagnosis of herpes simplex encephalitis instead of viral isolation.⁽¹⁶⁻¹⁸⁾

Acknowledgement

I would like to thank Dr. Somsak Pakdewongse,

Department of Communicable Diseases Control (Bangrak VD center, VD Division), Ministry of Public Health, Bangkok 10330, Thailand for his kindness in collecting the clinical specimens used throughout this study. This work was supported by Rajadapiseak Sompoj China Medical Board grant, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

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