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Evaluation of Biotin-Streptavidin Enzyme-Linked Immunosorbent Assay for Detection of Genital Herpes Simplex Virus Infection

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SUMMARY **A biotin – streptavidin enzyme – linked immunosorbent assay (B – SA ELISA) was evaluated for detection of herpes simplex virus (HSV) in clinical specimens which were cervico – vaginal swabs from 205 asymptomatic women and swabs from the genital lesions of 163 suspected patients. All specimens were also subjected to a conventional virus isolation in cell culture. A blocking B – SA ELISA had 100% specificity and 98% sensitivity compared with viral isolation from patients, but had only 40% sensitivity using specimens from asymptomatics. The conventional B – SA ELISA might also be used; it gave results corresponding to B – SA ELISA blocking test except for a single specimen which was considered a false positive.**

Various methods are available for detection of herpes simplex virus (HSV) in clinical specimens and they are different in sensitivity. The most classical is the isolation of infectious virus in susceptible cell cultures, but the drawback of this virological method is that it is labor intensive due to a requirement for daily observation of the infected culture and sometimes takes 5-7 days to complete. The assay could be accelerated, yet remains as sensitive as cell culture alone, by staining the infected cells after the viral antigens had been amplified during a short period of growth in culture.[1-3] Identification of physical HSV particles by electron microscopy using negative staining[4] is rapid but requires instrumentation not available in most virus laboratories. Indirect methods for identification of HSV were also described. These include direct staining of tissue scraped from the lesions[5] and the use of a solid-phase enzyme-linked immunosorbent assay (ELISA) to reveal the presence of HSV antigens in clinical swabs placed in transport medium.[6,7] These methods are more economical, practical and more simple than the conventional virus isolation for large scale studies. We therefore evaluated circumstances in which an ELISA could replace virus isolation by performing a double antibody sandwich ELISA using biotin-labelled antibody and streptavidin peroxidase (B-SA ELISA). This method has been considered the most sensitive presently available. Specimens from symptomatics and asymptomatics (no herpetic lesions) were included in this study.

MATERIALS AND METHODS

Specimens

Clinical materials were collected during September, 1985-April, 1986. They were cervico-vaginal swabs from women without any signs or symptoms of genital herpes, who attended the gynecological clinic of Ramathibodi Hospital, Bangkok, for an ordinary examination. The others were swabs from the suspected herpetic lesions at vulva, prepuce, glans penis, cervix, shaft, scrotal skin, coronus sulcus, subpreputial sac, labia majors and perineum of patients who consulted at the sexually-transmitted disease clinic, Bangrak Hospital, Bangkok. The swabs were placed in vials containing 1.5 ml transport medium (M 199 supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, N.Y., U.S.A.), 100 units penicillin and 100 μg streptomycin per ml). The specimens were kept frozen at -90°C within 6 hours and

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thawed at 37°C. Before HSV assay in Vero cell culture, the swab was squeezed against the container wall and discarded. The specimens were either filtered through 0.45 μm membrane or treated with 0.15 ml antibiotic solution (containing 500 units penicillin, 500 μg streptomycin, 50 μg kanamycin and 50 μg fungizone). This assay was carried out within a month. Specimens were considered positive for HSV if the characteristic cytopathic effects were observed within 5-7 days.

Of 710 specimens from asymptomatic women, only 5 were positive for HSV isolation. B-SA ELISA was carried out in these 5 HSV isolation-positive specimens and 200 other HSV isolation-negative specimens. Among more than 500 specimens from the symptomatics, 163 specimens were randomly selected for the studies, without any prior consideration on virus isolation results, of which 105 specimens were HSV positive.

Determination of virus titer

Titration of virus was carried out in Vero cells grown in M199 supplemented with 10% fetal bovine serum (GIBCO), 2 mM L-glutamine, 10 mM HEPES, 100 units penicillin and 100 μg streptomycin per ml (GM) in a 48-well tissue culture plate (Costar, Cambridge, Mass., U.S.A.). After viral adsorption, 0.5 ml of overlay medium (0.8% gum tragacanth in GM) was added and kept at 37°C in a 5% CO_2 incubator. The medium was removed at 5 days; the cells were stained with a mixture of 1% crystal violet and 10% formalin for 30 minutes, washed, air-dried and counted for the number of plaques. The titer of HSV was expressed as plaque-forming unit (PFU).

Purification of virus

HSV-2 (strain 186) was prepared in Vero cells infected with approximately 1 PFU of virus per cell in roller bottles. At 18-24 hours later, when more than 80% of the monolayers showed a typical cytopathic effect, the infected cells were scraped from the glass surface. Virus stock was made from this suspension after being frozen-thawed twice and the cell debris was removed.

To concentrate this virus, the infected cell suspension was centrifuged at 500 x g (International Equipment Company (IEC), Model PR-6, Needham Hts, Mass., U.S.A.) for 10 minutes. After the supernatant fluid was removed, the pellet was suspended in an equal volume of 1 mM Tris-HCl, pH 7.4,[8] frozen-thawed 3 times and Dounce homogenized for 4 strokes. The virus suspension was decanted after centrifugation at $1,100 \times g$ for 20 minutes followed by at $12,000 \times g$ (RC-5 Superspeed Refrigerated Centrifuge, Dupont Instrument, Sorval, Newtown, Conn., U.S.A.) for 30 minutes.

The purification of HSV-2 was carried out by density gradient centrifugation, using Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). The step was essentially as suggested by the company; a parallel tube containing Density Marker Beads instead of concentrated HSV-2 suspension was also included. The centrifugation was carried out in a Ti 60 rotor at $150,000 \times g$ (L8-70 Ultracentrifuge, Beckman, U.S.A.) for 30 minutes. The purified HSV-2, free of Percoll, was assayed as described above and the protein content was determined by the method of Lowry *et al.*[9] The preparation was then treated with 0.1% NP-40 for 90 minutes in an ice bath.

Sera

Rabbit immunoglobulin G fractions of HSV negative control sera and antisera to HSV-2 (MS strain) lot numbers 013 and 084, respectively, were obtained from DAKO (DAKOPATTS, Denmark).

A portion of the immunoglobulin fraction of rabbit antisera to HSV-2 (above) was linked to biotin by the method described by Nerurkar *et al.*[1] In brief, the immunoglobulin fraction was extensively dialyzed against phosphate-buffered saline (PBS) and then against 0.1 M $NaHCO_3$ and clarified by centrifugation at $300 \times g$ for 10 minutes. After the total protein content was adjusted to 1 mg/ml with PBS, pH 7.4, it was then mixed with freshly prepared biotin-succinimide ester (Sigma Chemical Company, St. Louis, Mo, U.S.A.; 1 mg per ml in dimethyl sulfoxide) at a volume ratio of 100:12 at room temperature for 4 hours and extensively dialyzed. The biotin-linked antibody was kept at -20°C in an equal volume of glycerol.

B-SA ELISA

Checkerboard titration as described by Voller *et al.*[10] was used to optimize the dose of capture and detecting antibodies and streptavidin peroxidase (Sigma); the latter had been suspended at 0.25 mg protein per ml in 50% glycerol in PBS.

Conventional B-SA ELISA was carried out in a 96-well Microwell plate (Nunc IIF, Denmark). Each well was coated with 100 μl of rabbit anti-HSV-2 immunoglobulin, diluted 1:200, in carbonate buffer, pH 9.6, at 4°C, overnight. The plate was then washed 3 times, 4 minutes each, with PBS, pH 7.4 and blocked with 150 μl per well of 2% bovine serum albumin (Sigma) at 4°C for at least 2 hours. The wells were washed 3 times with washing buffer containing 0.05% Tween 20 in PBS (PBS-T). Thereafter, 50 μl of specimen and 50 μl diluting buffer (1% BSA in PBS-T) was added in duplicate wells, kept for 18 hours at 4°C, if not indicated otherwise, and washed 3 times

before addition of 100 μl of biotinylated rabbit anti-HSV-2 (1:500). The plates were incubated at 37°C in a moisture chamber for 2 hours and washed 3 times, followed by addition of 100 μl of streptavidin peroxidase (1:1000) and incubated at 37°C for 1.5 hours, then washed again. One-hundred μl of 0.67% 1, 2-phenylene diamine dihydrochloride (DAKOPATTS) and 0.014% H_2O_2 was added and allowed to react at room temperature in the dark for 30 minutes. The reaction was terminated by an addition of 100 μl of 5N H_2SO_4. The optical density (OD) was assessed in a spectrophotometer at 490 nm (Minireader II, Dynatech Laboratory, Inc., Va., U.S.A.). Included in each plate were 6 negative (reagent) and 5 positive (diluted HSV-2 stock) control wells. The antigen was considered to be present if the averaged OD of the specimen was $\geqslant 2$ times the averaged OD of reagent controls.

A B-SA ELISA blocking test was also performed by an addition of 50 μl of rabbit anti-HSV-2, diluted 1:200, or 50 μl of negative control serum for ELISA (DAKOPATTS), diluted 1:50, as suggested by the company, instead of 50 μl diluent used in the conventional test. Percent blocking was calculated as

$$\frac{\text{OD normal sera-OD antisera}}{\text{OD normal sera}} \times 100.$$

This test was considered positive if blocking was at least 50%.

RESULTS

Optimization of incubation times for B-SA ELISA

Diluted HSV-2 stock was used as antigen in the conventional B-SA ELISA to determine the optimal incubation times for biotin-labelled rabbit anti-HSV-2 (detecting antibodies) and streptavidin peroxidase. The maximal binding periods were 120 and 90 minutes, respectively. However, the reaction times can be speeded up to 30 minutes each if more concentrated reagents are used.

Sensitivity of B-SA ELISA blocking test

Since the estimation of viral protein in HSV-2 stock and HSV isolation-positive specimens could not be performed due to the presence of other proteins of non-viral origin, NP-40 treated purified HSV-2 preparation was used as antigen to determine the sensitivity of this test. The antigen was serially diluted 2-fold and the reactions were carried out at 37°C for 2 hours or at 4°C for 18 hours. The results of the B-SA ELISA blocking test were similar for these 2 conditions up to a point which was apparently a borderline or limit of antigen detection. For example, with 14 ng of HSV-2 proteins, the B-SA ELISA blocking test was positive (53% ELISA blocking) for reaction at 4°C for 18 hours while it was negative (47% ELISA blocking) for reaction at 37°C for 2 hours (Fig. 1); to obtain a positive reaction with the 2-hour antigen incubation, at least double this amount or 28 ng of HSV-2 proteins were required.

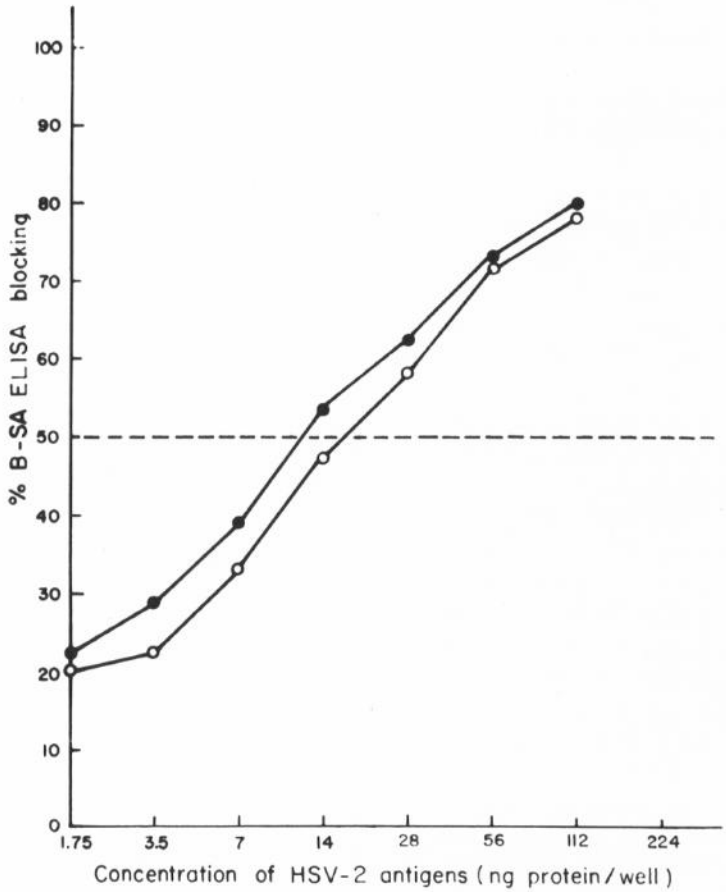

Fig. 1 Dose – response comparing HSV – 2 antigen detection by B – SA ELISA ELISA blocking test after incubation of antigens at 37°C for 2 hours (0 —— 0) and at 4°C for 18 hours (● —— ●). Dashed line indicates cutoff limit for B – SA ELISA blocking test.

Reaction times for antigens

To determine the effect of reaction time for antigens and capture antibodies, diluted HSV-2 stock was allowed to react at 37°C and the binding was observed at 30-minute intervals for 4 hours and compared to reaction at 4°C for 18 hours. If the latter was considered a maximal binding time, approximately 33, 67 and 91% of the antigens reacted during the first 30 minutes, 2 and 4 hours, respectively. Thus, positive conventional B-SA ELISA was detectable as early as 30 minutes of incubation for this dose of antigens used and there was a limit for antigen detection at each time-point, *i.e.*, antigens present in a small amount required a longer incubation period in order to demonstrate a positive reaction (data not included).

Detection of HSV antigens in clinical specimens by B-SA ELISA

Conventional B-SA ELISA was primarily used to detect HSV antigens in the clinical specimens; the OD control averaged from the multiple assays was 0.18 ± 0.04. Of 105 HSV isolation-positive specimens from symptomatic cases, 103 (98%) were positive by this test; another positive one was from an HSV isolation-negative specimen. In contrast, conventional B-SA ELISA could detect only 2 of 5 (40%) HSV isolation-positive specimens from asymptomatics (Table 1). All these 5 HSV isolation-positive and conventional B-SA ELISA-negative specimens (2 from symptomatics and 3 from asymptomatics) showed late cytopathogenic effect in culture, *i.e.*, starting 4 days after cultivation, and in some cases, in only 1 of duplicate cultures, or appearing as a few small patches of infected cells which required subcultivation to confirm the isolation.

To determine if conventional B-SA ELISA was specific for detection of HSV antigens, a B-SA ELISA blocking test was performed in all specimens. In order to calculate % B-SA ELISA blocking, some specimens which were shown to contain large amounts of antigen in the conventional B-SA ELISA were diluted 1:10 to 1:80 before assay. Examples of results obtained with 3 clinical specimens containing large amounts of infectious virus and probably non-infectious viral antigens are shown in Table 2. In this situation, the % B-SA ELISA blocking of the specimens was derived from the lowest dilutions which could be calculated. Virus isolation results and distribution of % ELISA blocking in specimens from symptomatics are also illustrated in Figure 2. We concluded that the results were similar to conventional B-SA ELISA except in one specimen (formerly HSV isolation-negative and conventional B-SA ELISA-positive) which was now negative for HSV antigens by the B-SA ELISA blocking test. The % B-SA ELISA blocking of the 2 specimens from asymptomatics (both HSV isolation and conventional B-SA ELISA-positive) were 55 and 64%.

Table 1 Comparison of HSV isolation, conventional B – SA ELISA and B – SA ELISA blocking test results of genital specimens

Source of specimens	No.	Virus isolation	Conventional B – SA ELISA[a]	B – SA ELISA blocking test[b]
Symptomatic	103	+	+	+
	2	+	–	–
	57	–	–	–
	1	–	+	–
Asymptomatic	2	+	+	+
	3	+	–	–
	200	–	–	–

[a]Specimen was considered positive if OD specimen ⩾ 2 times OD control.
[b]Specimen was considered positive if B – SA ELISA blocking value was ⩾ 50%

Table 2 Examples of B – SA ELISA results of some specimens from patients with genital herpes.

Specimen No.	Virus titer[a] (PFU/50 μl)	Dilution	B – SA ELISA[b] Conventional	B – SA ELISA blocking test (%)[c]
S 405	6.5×10^2	1:40	+	+ (90)
		1:80	+	+ (82)
		1:160	+	+ (73)
		1:320	+	+ (56)
S 411	5.5×10^2	1:10	+	+ (75)
		1:20	+	+ (66)
		1:40	–	– (49)
		1:80	–	– (45)
		1:160	–	– (26)
S 441	2.6×10^3	1:60	+	+ (73)
		1:120	+	+ (54)
		1:240	–	– (44)
		1:480	–	– (8)

[a]Determined from specimens that were frozen—thawed twice.
[b]Specimen was considered positive if OD specimen ⩾ 2 times OD control.
[c]Blocking value of ⩾ 50% was considered positive.

DISCUSSION

Recent advances in the management and treatment of genital HSV infection have led to a need for rapid and sensitive diagnosis of genital herpes. Although virus isolation in tissue culture does not detect all genital herpes cases,[11] it is still the most precise and accepted by most investigators; other alternative diagnostic methods are usually compared with this technic.[1,2,4,5,12-14] The results of the test in question can be expressed in terms of the sensitivity and specificity, which represent the fractions of virus isolation-positive and -negative specimens, respectively. Thus, a good test must overcome the problems of both false negatives and false positives, since these can create problems particularly in certain clinical settings where an immediate decision has to be made.[15]

Among the immunological diagnostic methods used to detect HSV, the ELISA is considered one of the most sensitive tests since it can detect antigens associated with not only infectious virus but also with non-infectious particles. Furthermore, binding of biotin and streptavidin in the B-SA ELISA serves as an aid in amplification of the sensitivity and gives a more specific assay than the enzyme-labelled antibody technic previously used.[16] The present studies revealed that the B-SA ELISA blocking test could detect 14-28 ng of HSV proteins under optimal conditions (Fig. 1). However, we realized that the results of virus isolation in cell culture should be considered primarily before any evaluation of B-SA ELISA for diagnosis of genital herpes was made.

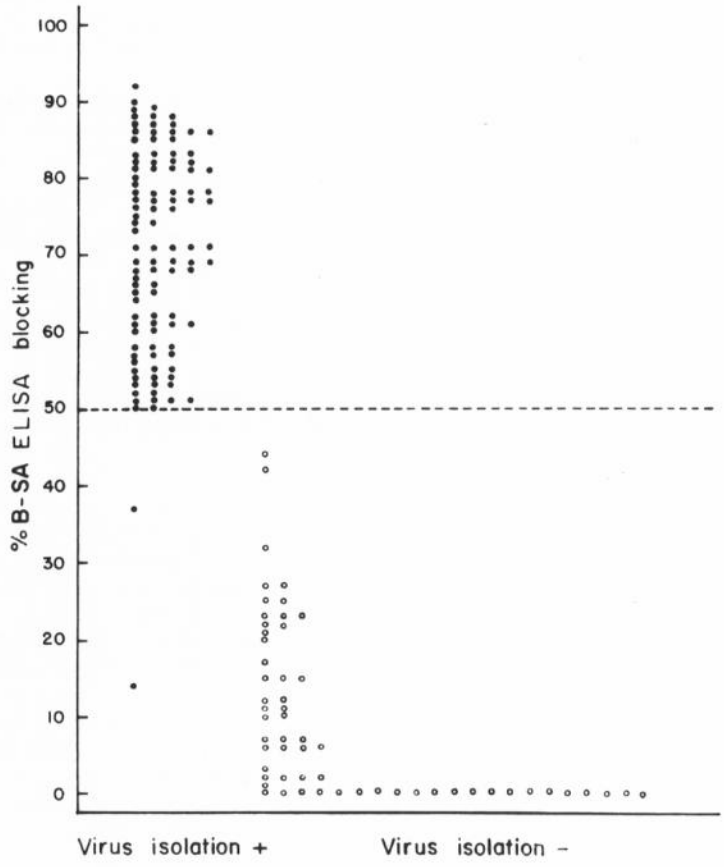

Fig. 2. Scattergram of percent B – SA ELISA blocking test derived from 105 virus isolation – positive specimens (●) and 58 virus isolation – negative specimens "O". Data were from the symptomatic cases. Dashed line indicates cutoff limit for B – SA ELISA blocking test.

In our studies, HSV was isolated from approximately 64% of the lesions (mostly vesiculars and ulceratives). The recovery rate was comparable to or higher than those reported by other investigators.[2,5,6,7,17,18] There was a very good correlation between a B-SA ELISA and virus isolation results (Table 1). In the conventional B-SA ELISA, 103 of 105 virus isolation-positive specimens were positive, while only 1 of 58 virus isolation-negative specimens from symptomatics was positive. In asymptomatics, only 2 of 5 virus isolation-positive specimens were positive, thus, giving only a 40% sensitivity, and there was no false positive. Furthermore, there was an agreement between conventional B-SA ELISA and B-SA ELISA blocking test results except in one specimen from the symptomatics (virus isolation-negative and B-SA ELISA-positive which was not detected in the blocking test). The high degree of sensitivity (98%) and specificity (100%) for ELISA blocking test in the symptomatics may be contributed by the polyclonal antibodies of various specificities used and a proper handling of the specimens before virus isolation. The latter probably explained why we were not able to detect specimens that were virus isolation-negative and B-SA

ELISA positive as noted by other investigators using a similar assay;[7] our procedure differed only in the substrate used and evaluation of positive result. The results in Table 2 suggested that the B-SA ELISA identified mostly non-infectious HSV antigens which might be present more abundantly in specimens swabbed from the active lesions than around asymptomatic shedding areas.

Although rabbit IgG anti-HSV-2 was used as capture and detecting antibodies in the B-SA ELISA, we assumed that this test identified both HSV-2 and the cross-reacting HSV-1. However, one might argue that the virus isolation-positive and B-SA ELISA-negative specimens (2 from the symptomatics and 3 from the asymptomatics) were HSV-1 strains and might not react well with the capture or detecting antibody. However, this is not the case. HSV typing of these isolates either by use of type-specific commercially available monoclonal antibodies[19] (Cultureset, Ortho Diagnostic Systems, Raritan, NJ, USA) or by restriction endonuclease analysis of their DNA genome[20] indicated that all were type 2.

We then concluded that the B-SA ELISA appeared suitable for detection of HSV only in genital swabs from the lesions, but was not satisfactory for screening asymptomatics in whom excretion of virus or antigens may be very low. Moreover, the conventional B-SA ELISA seemed economical and sufficiently specific. The test could be accomplished within 4 hours if the antibody-coated plate was available and proper concentrations of reagents were used.

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