

RESEARCH ARTICLE

Comparison of commercial methods of immunoblot, ELISA, and chemiluminescent immunoassay for detecting type-specific herpes simplex viruses-1 and -2 IgG

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Background: Serology for type-specific herpes simplex virus (HSV) is based on the use of the respective glycoprotein G (gG).

Methods: Chemiluminescent immunoassay (CLIA; BIO-FLASH[®], Biokit, Spain), ELISA (HerpeSelect[®], Focus, USA), and immunoblot (IB; Virotech, Germany) for detecting HSV-1- and HSV-2-specific IgG were compared using 384 serum samples received for HSV serology. The samples were classified as positive or negative according to a consensus criterion.

Results: For HSV-1, 262 samples were positive and 118 were negative (four samples were unclassifiable). IB showed agreement, sensitivity, and specificity values of 98.68%, 98.47% and 99.15%, respectively. The corresponding figures for CLIA and ELISA were 98.95%, 99.24% and 98.31%, and 98.16%, 99.62% and 94.92%, respectively. For HSV-2, 106 samples were positive and 278 were negative. Agreement, sensitivity, and specificity of IB were 99.48%, 98.11%, and 100%, respectively. The corresponding figures for CLIA and ELISA were 99.48%, 99.06% and 99.64%, and 98.18%, 99.06% and 97.84%, respectively.

Conclusion: The three methods showed excellent and equivalent performance characteristics for the detection of type-specific IgG to HSV-1 and HSV-2.

KEYWORDS

chemiluminescent immunoassay, enzyme immunoassay, herpes simplex virus type 1, herpes simplex virus type 2, immunoblot

1 | INTRODUCTION

There are two types of herpes simplex virus (HSV): HSV-1 and HSV-2. Both viruses cause an initial infection in peripheral tissue followed by the establishment of a latent infection in nervous cells from the regional sensorial ganglia. However, they show different tropism: HSV-1 infects the oral mucosa, causing an orolabial disease, and establishing latency in the trigeminal ganglion; HSV-2 produces a genital disease (genital herpes), establishing latency in the lumbosacral ganglion. However, the epidemiology of genital herpes seems to have changed in recent years, whereby a significant number of genital infection cases are currently caused by HSV-1.¹ The seroprevalence of both types of

HSV differs; for example, in Spanish women, the values of HSV-1 and HSV-2 are, respectively, 78.6% and 3.5%.² Thus, the differentiation of type-specific HSV responses is an important issue for clinical laboratories when they attempt to characterize herpes infections. HSV serological diagnosis has for years used antigen extracts of HSV-infected cells, but this approach does not allow type-specific serological responses to be differentiated independently of the virus used to obtain the antigen.³ Furthermore, the cross-reactivity between HSV-1 and HSV-2 and other herpesviruses, especially VZV, is a serious hindrance to the characterization of the specific serological response.⁴ HSV-1 and HSV-2 glycoprotein G (gG; respectively, gG1 and gG2) have been recognized as being type-specific for the corresponding virus,

and show good discrimination of antibodies to HSV-1 and HSV-2.⁵ Serological assays based on the use of gG1 or gG2 as the antigen have been developed to identify type-specific antibodies.

Currently, there are several commercial assays available that use gG for detecting type-specific HSV-IgG, showing appropriate performance characteristics, including ELISA,^{6,7} immunochromatography (IC),⁸ immunoblot (IB) and immunoplexed assays.⁹ The aim of the study reported in this article was to comparatively evaluate ELISA, IB, and chemiluminescent immunoassays (CLIAs) recently developed for identifying specific IgG responses to HSV-1 and HSV-2.

2 | MATERIALS AND METHODS

The study included 384 samples, received in the Serology Laboratory of the National Center for Microbiology, for the purpose of examining serology against HSV. Samples were stored at -20°C until use.

The assays compared were indirect ELISA (HerpeSelect[®]1 ELISA IgG, and HerpeSelect[®]2 ELISA IgG) from Focus Diagnostics (Cypress, CA, USA), CLIA (BIO-FLASH[®] HSV-1 IgG and BIO-FLASH[®] HSV-2 IgG) from Biokit SA (Lliçà d'Amunt, Barcelona, Spain), and IB (HSV LINE IgG Line Immunoblot) from Sekisui Virotech (Rüsselsheim, Germany).

HerpeSelect[®] ELISA IgG tests were done in an ELISA device (Behring ELISA Processor III [BEP III], Siemens Healthcare, Marburg, Germany). Briefly, samples and controls diluted 1:101 were inoculated in polystyrene microwells containing gG1 or gG2 (in the HSV-1 and HSV-2 assays, respectively), and incubated for 1 hour at room temperature. Unbound reactants were removed by washing (three cycles) and peroxidase-conjugated anti-human IgG was added and incubated, as above. Excess conjugate was removed by washing. Enzyme substrate and chromogen were subsequently added, and the color was allowed to develop. After stopping the reaction, the resultant color change was quantified by a spectrophotometric reading of optical density (OD). Sample OD readings were compared with reference cut-off values (the mean of three determinations of a control) to calculate the results. The samples were classified as positive if the OD sample/OD cut-off was >1.1 , and as negative if the ratio was <0.9 . Samples showing ratios ≥ 0.9 and ≤ 1.1 were defined as indeterminate, retested in duplicate, and finally classified as positive (if both replicates were positive), negative (if both replicates were negative) or indeterminate (if at least one replicate was indeterminate).

BIO-FLASH[®] HSV-1 IgG and BIO-FLASH[®] HSV-2 IgG tests were performed automatically with the BIO-FLASH[®] instrument (Biokit SA). Briefly, paramagnetic particles coated with recombinant gG1 or recombinant gG2 (in the HSV-1 and HSV-2 assays, respectively) were incubated with undiluted sample. If present, specific IgG bound to the antigen. A wash step was performed to remove unbound material. An anti-IgG (human) isoluminol conjugate was added and after another wash, as above, the reagents that trigger the chemiluminescent reaction were added. The emitted light, measured as relative light units, is directly proportional to the amount of specific IgG present in the sample. To generate a calibration curve, the BIO-FLASH[®] device uses a four-parameter logistic curve fit data reduction method. This

curve is lot-dependent and is stored in the instrument. With the assay of calibrators the predefined curve is transformed into a working calibration curve. BIO-FLASH results are reported as the signal/cut-off (S/CO) ratio; samples with $S/CO \geq 1.0$ were considered positive.

Finally, the samples were analyzed by IB in an IB device (Profiblot 4B; Tecan, Grödig, Austria). The IB assay determines IgG against a common HSV antigen (native), and gG1 (recombinant, species-specific glycoprotein of the Baculovirus system) and gG2 (affinity chromatography-purified species-specific glycoprotein). Samples were tested at 1:101 dilution and identified as being positive for each antigen if they had an intensity greater than those of the gG2 band of a cut-off control included in the kit.

To complete the determination of the serological profile of discrepant samples, HSV-IgG and VZV IgG were assayed by indirect ELISA (Enzygnost; Siemens Healthcare) in a BEP III device.

All serological determinations were carried out following the manufacturer's instructions strictly.

Samples were classified as positive or negative on the basis of a consensus result (at least two coincident positive or negative results with two different techniques).

To calculate sensitivity and specificity of the methods compared, indeterminate results were considered as the most adverse conditions. In other words, when the reference result was negative, an indeterminate result was considered to be positive; conversely, when the reference result was positive, the indeterminate one was considered to be negative.

Statistics were calculated by using Analyse-it version 4.60 (Analyse-it Software, Ltd., Leeds, UK). Kappa statistics were used to calculate the extent of agreement, and the 95% confidence interval (CI), of the determinations made with IB, BIO-FLASH[®] and HerpeSelect[®] for the HSV-1 and HSV-2 assays. To interpret the results, the following kappa values were considered: <0 , less than chance agreement; 0.01-0.20, slight agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, substantial agreement; 0.81-0.99, almost perfect agreement.¹⁰ Cases with any equivocal or indeterminate results were excluded from the kappa analysis.

3 | RESULTS

3.1 | HSV-1

When assayed by IB, 260 samples were positive and 124 were negative. 264 samples gave a positive result with CLIA, one was indeterminate and 119 were negative; when tested by ELISA, 261 samples were positive, 112 were negative and the remaining 11 were indeterminate. A total of 364 samples showed agreement in the three assays, of which 109 had a negative result and 255 had a positive results. Results from the remaining 20 samples were discrepant (Table 1). Four samples were considered unclassifiable, since they gave a different result in each assay (#6, #76, and #9) or an indeterminate result in two assays (#63). Of the samples classified as positive, four were classified as false negatives by IB (#26, #33, #37, and #77), two by CLIA (#64 and #69), and one (#70, with an indeterminate result) by ELISA. Of the samples finally classified as negative, false positives

TABLE 1 Comparison of HSV-1 assays. Samples showing discrepant results^a

Sample	IB-HSV1/2	CLIA	ELISA	Final classification	IgG HSV ^b	IgG VZV ^b	VZV/HSV ^c
6	NEG/NEG	POS	IND	UNCLASSIFIABLE	<1	66	>66
76	NEG/NEG	POS	IND	UNCLASSIFIABLE	<1	66	>66
9	POS/POS	NEG	IND	UNCLASSIFIABLE	70	32	0.5
63	NEG/NEG	IND	IND	UNCLASSIFIABLE	2.9	<1	<0.3
26	NEG/NEG	POS	POS	POSITIVE	4.8	52	10.8
33	NEG/NEG	POS	POS	POSITIVE	5.2	42	8.1
37	NEG/NEG	POS	POS	POSITIVE	3.4	42	12.4
77	NEG/NEG	POS	POS	POSITIVE	19.1	42	2.2
64	POS/POS	NEG	POS	POSITIVE	61	42	0.7
69	POS/NEG	NEG	POS	POSITIVE	6.1	26	4.3
70	POS/NEG	POS	IND	POSITIVE	4.8	36	7.5
81	POS/NEG	NEG	NEG	NEGATIVE	2.9	38	13.1
18	NEG/NEG	POS	NEG	NEGATIVE	6.1	34	5.6
53	NEG/NEG	POS	NEG	NEGATIVE	<1	66	>66
21	NEG/NEG	NEG	IND	NEGATIVE	4.8	52	10.8
27	NEG/NEG	NEG	IND	NEGATIVE	10.9	66	6.1
31	NEG/NEG	NEG	IND	NEGATIVE	<1	42	>42
54	NEG/NEG	NEG	IND	NEGATIVE	3.1	40	12.9
2	NEG/POS	NEG	IND	NEGATIVE	14.3	28	2
11	NEG/POS	NEG	IND	NEGATIVE	100	34	0.3

POS, positive; IND, indeterminate; NEG, negative.

^aDiscrepancies after final classification of samples are in bold.

^bResults expressed as the ratio of samples absorbance/cut-off (230 [titer] for HSV; and 50 [mIU/mL] for VZV).

^cExpressed as the IgG VZV/IgG HSV ratio.

were determined in one sample by IB (#81), two samples by CLIA (#18 and #53) and six samples by ELISA (#21, #27, #31, #54, #2, and #11, all with indeterminate result).

Thus, after the final classification of samples, 262 were positive and 118 negative, the remaining four not being classifiable. Results obtained with IB, CLIA, and ELISA against the reference consensus criterion are given in Table 2. Agreement, sensitivity, and specificity of IB were 98.68% (95% CI: 96.9% to 99.5%), 98.47% (95% CI: 96.0% to 99.6%) and 99.15% (95% CI: 94.9% to >99.9%); the corresponding figures for CLIA were 98.95% (95% CI: 97.2% to 99.7%), 99.24% (95% CI: 97.1% to >99.9%) and 98.31% (95% CI: 93.7% to >99.9%), and for ELISA were 98.16% (95% CI: 96.2% to 99.2%), 99.62% (95% CI: 97.7% to >99.9%), and 94.92% (95% CI: 89.1% to 97.9%). Kappa values were 0.98 for IB, 0.98 for CLIA and 1 for ELISA IgG, indicating almost perfect agreement, although 11 samples showing indeterminate results were excluded from the analysis.

The 20 discrepant samples were tested for VZV IgG and HSV-IgG by ELISA. The positive (in CLIA) and indeterminate (in ELISA) results detected in samples #6 and #76 (Table 1), considered to be unclassifiable, could have been the result of a strong response to VZV IgG in the absence of HSV-IgG. The same happened with samples #53 (single positive result with CLIA) and #31 (single indeterminate result with

TABLE 2 Performance characteristics of the three methods for HSV-1 IgG after classification of samples

	Reference	
	Positive	Negative
Immunoblot		
Positive	258	1
Negative	4	117
CLIA		
Positive	260	2
Negative	2	116
ELISA		
Positive	261	0
Indeterminate	1	6
Negative	0	112

Immunoblot: Agreement: 98.68%, 95% CI: 96.9% to 99.5%; Sensitivity: 98.47%, 95% CI: 96.0% to 99.6%; Specificity: 99.15%, 95% CI: 94.9% to >99.9%.

CLIA: Agreement: 98.95%, 95% CI: 97.2% to 99.7%; Sensitivity: 99.24%, 95% CI: 97.1% to >99.9%; Specificity: 98.31%, 95% CI: 93.7% to >99.9%.

ELISA: Agreement: 98.16%, 95% CI: 96.2% to 99.2%; Sensitivity: 99.62%, 95% CI: 97.7% to >99.9%; Specificity: 94.92%, 95% CI: 89.1% to 97.9%.

ELISA) among the group of negatives. The single indeterminate result obtained in ELISA in samples #2 and #11, positive for HSV-2 in IB and showing a high titer of HSV-IgG could have been the result of the cross-reactivity between HSV-1 and HSV-2. In the remaining samples in this group, all those with a positive result in HSV-IgG and a single positive result with IB (#81), with CLIA (#18) or that were indeterminate with ELISA (#21, #27, #54), had a VZV IgG titer that was much higher than that for HSV-IgG (Table 1).

3.2 | HSV-2

When assayed by IB 104 samples were positive and 280 were negative. With CLIA, 106 samples gave a positive result, one was indeterminate and 277 were negative; with ELISA, 106 were positive, five were indeterminate and 273 were negative. 373 samples showed agreement in the three assays, of which 271 and 102 had negative and positive results, respectively. Discrepant results were obtained in the remaining 11 samples (Table 3). Four samples were finally classified as positive; two samples (#94 and #165) by IB, one by CLIA (#56, with an indeterminate result), and one by ELISA (#112) were classified as false negatives. With respect to the samples classified as negative, a false-positive result was obtained by CLIA in one sample (#36), in six samples by ELISA, and five with an indeterminate result (#55, #146, #181, #90, and #92), the remaining sample being positive (#65). No false-positive results were obtained by IB.

Thus, after the final classification of samples, 106 were positive and 278 were negative. Results obtained by IB, CLIA, and ELISA are given in Table 4. Agreement, sensitivity, and specificity of IB were 99.48% (95% CI: 98.1% to 99.9%), 98.11% (95% CI: 93.4% to 99.5%) and 100% (95% CI: 98.6% to >99.9%). The corresponding figures for CLIA and ELISA were 99.48% (95% CI: 98.1% to 99.9%), 99.06% (95% CI: 94.8% to 99.8%) and 99.64% (95% CI: 98.0% to 99.9%), and 98.18% (95% CI: 96.3% to 99.1%), 99.06% (95% CI: 94.8% to 99.8%) and 97.84% (95% CI: 95.4% to 99.0%), respectively. Kappa values

TABLE 4 Performance characteristics of the three methods for HSV2 IgG after classification of samples

	Reference	
	Positive	Negative
Immunoblot		
Positive	104	0
Negative	2	278
CLIA		
Positive	105	1
Indeterminate	1	
Negative		277
ELISA		
Positive	105	1
Indeterminate		5
Negative	1	272

Immunoblot: Agreement: 99.48%, 95% CI: 98.1% to 99.9%; Sensitivity: 98.11%, 95% CI: 93.4% to 99.5%; Specificity: 100%, 95% CI: 98.6% to >99.9%.

CLIA: Agreement: 99.48%, 95% CI: 98.1% to 99.9%; Sensitivity: 99.06%, 95% CI: 94.8% to 99.8%; Specificity: 99.64%, 95% CI: 98.0% to 99.9%.

ELISA: Agreement: 98.18%, 95% CI: 96.3% to 99.1%; Sensitivity: 99.06%, 95% CI: 94.8% to 99.8%; Specificity: 97.84%, 95% CI: 95.4% to 99.0%.

were 0.98 for IB, 0.99 for CLIA and 0.98 for ELISA IgG, indicating an almost perfect agreement, although samples showing indeterminate results were excluded from the analysis.

The 11 discrepant samples were analyzed for VZV IgG and HSV-IgG by ELISA (Table 3). Among the negative samples, the single positive result with CLIA (sample #36), and the indeterminate result with ELISA (#55) could have been caused by the cross-reactivity to VZV. The remaining samples with a single indeterminate (#146, #181, #90, #92) or positive (#65) result with ELISA could be better explained by cross-reaction between HSV-1 and HSV-2.

TABLE 3 Comparison of HSV-2 assays. Samples showing discrepant results^a

Sample	IB-HSV1/2	CLIA	ELISA	Final classification	IgG HSV ^b	IgG VZV ^b	VZV/HSV ^c
94	POS/NEG	POS	POS	POSITIVE	9.6	24	2.5
165	POS/NEG	POS	POS	POSITIVE	157	6.2	0.04
56	POS/POS	IND	POS	POSITIVE	126	42	0.3
112	POS/POS	POS	NEG	POSITIVE	4.3	42	9.8
36	NEG/NEG	POS	NEG	NEGATIVE	4.8	42	8.8
55	NEG/NEG	NEG	IND	NEGATIVE	<1	24	>24
146	POS/NEG	NEG	IND	NEGATIVE	8.4	38	4.5
181	POS/NEG	NEG	IND	NEGATIVE	157	22	0.1
90	POS/NEG	NEG	IND	NEGATIVE	87	8	0.1
92	POS/NEG	NEG	IND	NEGATIVE	96	42	0.4
65	POS/NEG	NEG	POS	NEGATIVE	96	42	0.4

POS, positive; IND, indeterminate; NEG, negative.

^aDiscrepancies after final classification of samples are in bold.

^bResults expressed as the ratio of samples absorbance/cut-off (230 [titer] for HSV; and 50 [mIU/mL] for VZV).

^cExpressed as the IgG VZV/IgG HSV ratio.

4 | DISCUSSION

The specific identification of antibodies to HSV-1 and HSV-2 is only possible by means of assays based on the use of type-specific gG.¹¹ Several commercial assays have been developed for testing HSV-1 and HSV-2-specific antibodies. When commercial assays using recombinant antigens were compared with others based on inactivated viral particles, the specificity was improved from 68%–72% to 96% for HSV-1 and from 61%–85% to 100% for HSV-2.¹²

Some of the commercial assays using recombinant antigens have been evaluated, including ELISA for HSV-1 and HSV-2^{6,13,14} or HSV-2 alone,^{15,16} lateral flow IC,^{8,17–19} or multiplex immunoassays for HSV-1 and HSV-2.^{9,20} Western blot is currently recognized as the gold standard for HSV type-specific serology,²¹ but the technique is difficult to standardize and is not available in most laboratory settings. To resolve this, different approaches to establishing the performance characteristics of new assays for HSV-specific serology were applied. In the case of HSV-2, serum samples from cultured proven cases were employed to calculate the sensitivity¹⁶ and samples from children were used to calculate the specificity.^{13,16,17} In the present study, we used a consensus criterion of the three compared methods to derive a final classification of discrepant samples. Comparison of all the assays yielded excellent levels of agreement, sensitivity, and specificity compared with the consensus result.

An important aspect of defining the performance characteristics of assays for type-specific HSV serology is the well-established cross-reactivity between HSV-1 and HSV-2 and other herpesviruses, especially VZV, which affects the levels of total antibodies or IgG in serum or cerebrospinal fluid^{4,22} or to IgM.²³ To explore the impact of such cross-reactions in this comparative evaluation, we tested the samples showing discrepant results in the comparative HSV-1 and HSV-2 assays in an ELISA for VZV IgG. We obtained discrepant results in 20 samples (5.20%) in the HSV-1 assays, and 11 (2.86%) in the HSV-2 assays. The results affecting the specificity of the assays could be evaluated on the basis of the cross-reactivity between HSV-1 and HSV-2, and VZV. For HSV-1-discrepant samples, VZV seems to be an important cause of cross-reactivity, as the reactivity to this virus is much higher than that to HSV in other samples (Table 1). This seems to be true for samples #6 and #76 (grouped as unclassifiable), #31 (single indeterminate result with ELISA), #53 (single positive result with CLIA), #18 (single positive result with CLIA), #81 (single positive result with IB), and #21, #27, #54 (all indeterminate with ELISA; Table 1). On the other hand, the single indeterminate result obtained by ELISA in samples #2 and #11, which was positive for HSV-2 with IB and showed a high titer of HSV-IgG, could have been a consequence of the cross-reactivity between HSV-1 and HSV-2 (Table 1).

For HSV-2, samples #55 (indeterminate with ELISA) and #36 (positive with CLIA) seem to be caused by cross-reactivity with VZV; in the remaining negative samples with single indeterminate (#146, #181, #90, #92) or positive (#65) results with ELISA, the discrepancies could be explained by cross-reaction between HSV-2 and HSV-1 (Table 3).

These results strongly suggest that discrepant results with the type-specific methods may be explained on the basis of antigenic

cross-reactivity with highly positive IgG VZV samples, as has previously been described,^{4,22} or between HSV-1 and HSV-2.

Another possible explanation for the discrepant results is that inadequate cut-off value was used in the assays. Previous reports suggest that increasing the cut-off of the HSV-2 ELISA used in this study would have improved the specificity of the assay; this seems especially to be the case for samples from sub-Saharan individuals.²⁴ To explore this further, the results were recalculated considering cut-off values of 3.0 and 3.5 for the ELISA assay. For a cut-off of 3.0, the sensitivity and specificity were 94.7% and 100% (for HSV-1), and 89.6% and 98.9% (HSV-2); for a cut-off of 3.5, the corresponding figures were 93.1% and 100% (HSV-1), and 85.8% and 98.9% (HSV-2). Accordingly, for the HSV-2 assay, the specificity increased slightly (from 97.84% to 98.9%), but the sensitivity was substantially decreased (from 99.06% to 85.8%; data not shown).

This study was designed to compare the methods for determining HSV-1 and HSV-2 IgG in samples received in a serology context. Clinical and epidemiological information was not available for most samples, so it was not possible to consider factors that may have affected the characteristics of the tests, such as the presence of acute herpes infection, or the prevalence of some other infections (eg, HIV).

In conclusion, comparing the three methods showed them all to have excellent and comparable performance characteristics for detecting type-specific IgG to HSV-1 and HSV-2. The IB method is advantageous in that it permits the simultaneous determination of HSV-1 and HSV-2 IgG in a single assay, and BIO-FLASH[®] has the advantages of a shorter handling time and no requirement to aliquot specimens before assaying them. However, ELISA has the drawback of generating a relatively large number of indeterminate results, making it difficult to characterize samples correctly.

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