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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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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ABSTRACT

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.

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 crossreactivity 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 paper was to comparatively evaluate ELISA, IB and chemiluminescent immunoassays (CLIAs) recently developed for identifying specific IgG responses to HSV-1 and HSV-2.

MATERIAL 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 BIOFLASH® 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 h 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 ≥ 1.0 were considered positive.

Finally, the samples were analyzed by IB in an IB device (Profiblot 4B, Tecan). 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. 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.

RESULTS

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. 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 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 shown in Table 2.

Agreement, sensitivity and specificity of IB were 98.68% (95% CI: 96.9-99.5%), 98.47% (95% CI: 96.0-99.6%) and 99.15% (95% CI: 94.9->99.9%); the corresponding figures for CLIA were 98.95% (95% CI: 97.2-99.7%), 99.24% (95% CI: 97.1->99.9%) and 98.31% (95% CI: 93.7->99.9%), and for ELISA were 98.16% (95% CI: 96.2-99.2%), 99.62% (95% CI: 97.7->99.9%), and 94.92% (95% CI: 89.1-97.9%). 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 due to 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 ELISA) amongst 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 due to the crossreactivity 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).

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 shown in Table 4. Agreement, sensitivity and specificity of IB were 99.48% (95% CI: 98.1-99.9%), 98.11% (95% CI: 93.4-99.5%) and 100% (95% CI: 98.6->99.9%). The corresponding figures for CLIA and ELISA were 99.48% (95% CI: 98.1-99.9%), 99.06% (95% CI: 94.8-99.8%) and 99.64% (95% CI: 98.0-99.9%), and 98.18% (95% CI: 96.3-99.1%), 99.06% (95% CI: 94.8-99.8%) and 97.84% (95% CI: 95.4-99.0%), respectively. Kappa values 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). Amongst the negative samples, the single positive result with CLIA

(sample #36), and the indeterminate result with ELISA (#55) could have been due to crossreactivity to VZV. The remaining samples with a single indeterminate (#146, #181, #90, #92) or positive (#65) result with ELISA could be better explained by crossreaction between HSV-1 and HSV-2.

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, 18, 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 sensitivity (16) 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 crossreactivity between HSV-1 and HSV-2 and other herpesviruses, especially VZV, which affects levels of total antibodies or IgG in serum or cerebrospinal fluid (4, 22) or to IgM (23). In order to explore the impact of such crossreactions 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 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 crossreactivity between HSV-1 and HSV-2, and VZV. For HSV-1-discrepant samples, VZV seems to be an important cause of crossreactivity, since 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 crossreactivity 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 crossreactivity 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 crossreaction 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 crossreactivity 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 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 (e.g., 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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Table 1. Comparison of HSV-1 assays. Samples showing discrepant results*.

Sample	IB-HSV1/2	CLIA	ELISA	Final classification	IgG HSV#	IgG VZV#	VZV/ HSV♣
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

*Discrepancies after final classification of samples are in bold. POS: positive; IND: indeterminate; NEG: negative; #results expressed as the ratio of samples absorbance/cut-off (230 [titer] for HSV; and 50 [mIU/ml] for VZV); ♣expressed as the IgG VZV/IgG HSV ratio.

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
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%			

		Reference	
		POSITIVE	NEGATIVE
CLIA			
	POSITIVE	260	2
	NEGATIVE	2	116
Agreement: 98.95%, 95% CI: 97.2 - 99.7%			
Sensitivity: 99.24%, 95% CI: 97.1 - >99.9%			
Specificity: 98.31%, 95% CI: 93.7 - >99.9%			

		Reference	
		POSITIVE	NEGATIVE
ELISA			
	POSITIVE	261	0
	INDETERMINATE	1	6
	NEGATIVE	0	112
Agreement: 98.16%, 95% CI: 96.2 - 99.2%			
Sensitivity: 99.62%, 95% CI: 97.7 - >99.9%			
Specificity: 94.92%, 95% CI: 89.1 - 97.9%			

Table 3. Comparison of HSV-2 assays. Samples showing discrepant results*.

Sample	IB-HSV1/2	CLIA	ELISA	Final classification	IgG HSV#	IgG VZV#	VZV/HSV♣
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

*Discrepancies after final classification of samples are in bold. POS: positive; IND: indeterminate; NEG: negative; #results expressed as the ratio of samples absorbance/cut-off (230 [titer] for HSV; and 50 [mIU/ml] for VZV); ♣expressed as the IgG VZV/IgG HSV ratio.

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
Agreement: 99.48%, 95% CI: 98.1 - 99.9%			
Sensitivity: 98.11%, 95% CI: 93.4 - 99.5%			
Specificity: 100%, 95% CI: 98.6 - >99.9%			

		Reference	
		POSITIVE	NEGATIVE
CLIA			
	POSITIVE	105	1
	INDETERMINATE	1	
	NEGATIVE		277
Agreement: 99.48%, 95% CI: 98.1 - 99.9%			
Sensitivity: 99.06%, 95% CI: 94.8 - 99.8%			
Specificity: 99.64%, 95% CI: 98.0 - 99.9%			

		Reference	
		POSITIVE	NEGATIVE
ELISA			
	POSITIVE	105	1
	INDETERMINATE		5
	NEGATIVE	1	272
Agreement: 98.18%, 95% CI: 96.3 - 99.1%			
Sensitivity: 99.06%, 95% CI: 94.8 - 99.8%			
Specificity: 97.84%, 95% CI: 95.4 - 99.0%			