Paper I

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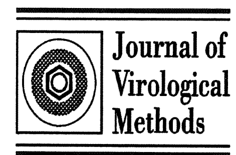

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A branched, synthetic oligopeptide corresponding to a region of glycoprotein G of HSV-1 reacts sensitively and specifically with HSV-1 antibodies in an ELISA

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Abstract

Herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2), which are common worldwide, are so similar that antibodies directed against one serotype may crossreact with antigens from the other one. Methods for specific detection of antibodies against HSV-1 or HSV-2 are based upon the antigenicities of glycoproteins G. However, due to the cost, the available commercial methods may not readily be used in developing countries. A different enzyme-linked immunosorbent assay (ELISA) method, based upon a synthetic oligopeptide corresponding to an immunogenic region in glycoprotein G of HSV-2, has been used recently and successfully for detection of HSV-2 antibodies. In the present study, the sequences of a newly identified immunogenic and type-specific region in glycoprotein G of HSV-1 was used to synthesize three different, branched oligopeptides. The performances of these peptides in an ELISA were investigated by testing Scandinavian and African sera which were characterized by commercial ELISA and Western blotting methods and divided into four groups either lacking HSV antibodies, containing antibodies against one or the other virus, or against both types. The peptide which corresponded in sequence to the immunodominant region was as specific and sensitive by an ELISA as were the commercial methods. The method is inexpensive and reliable.
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Keywords: HSV-1 serodiagnosis; Branched oligopeptide; ELISA

1. Introduction

Herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2) replicate in epithelial cells at the portal of entry in humans and cause mucocutaneous lesions in the oro-facial and genital regions. The viruses establish latent infections in sensory and autonomic ganglia from which reactivation can occur. HSV may infect the meninges and brain to cause meningitis and encephalitis. In contrast to HSV-1, HSV-2 spreads almost exclusively by sexual contact and is the most prevalent cause of genital herpes (Corey et al., 1983). In recent years, however, genital infections by HSV-1 have become increasingly important in several countries (Koelle et al., 1992; Ross et al., 1993; Tayal and Pattman, 1994; Christie et al., 1997; Corey and Handsfield, 2000; Lafferty et al., 2000; Nilsen and Myrmel, 2000). Many reports indicate that there is an association between genital herpes and HIV infection (Hook et al.,

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1992; Stewart et al., 1995; Morse et al., 1997; Cohen, 1998; Chen et al., 2000).

HSV-1 infections are common all over the world. Thus, between 60 and 90% of the adult population in Europe and USA has HSV-1 antibodies (Smith and Robinson, 2002). The prevalence of HSV-2 among adults varies between 20 and 90%, depending upon country, region within the country and subgroups of population (Smith and Robinson, 2002).

The various methods for diagnosis of HSV infections serve different purposes. Positive lesion specimen tests, demonstrated either by polymerase chain reaction (PCR) or by virus isolation and detection of specific virus antigens, document a current infection. Detection of specific antibodies indicates seroconversion following a recent infection or an immunological response to an earlier infection.

HSV-1 and HSV-2 are so similar in their genomes and proteins that most antibodies raised against one type cross-react with the other type. Differentiation between immunological responses to HSV-1 and HSV-2 was therefore difficult until glycoprotein G of HSV-2 (gG-2) was identified (Marsden et al., 1984; Roizman et al., 1984) and shown to be a type-specific antigen. Glycoprotein G-2 is now widely accepted and used for serological diagnosis of HSV-2 infection (Ashley et al., 1988; Sullender et al., 1988; Parkes et al., 1991; Sanchez-Martinez et al., 1991; Ho et al., 1992; Bergström and Trybala, 1996; Hashido et al., 1997; Ashley et al., 1998). Similarly, glycoprotein G of HSV-1 (gG-1) is used in an enzyme immunoassay for detection of HSV-1 antibodies (Ashley, 2001; Prince et al., 2000). Western blotting, in which the antigens are derived from cells infected with HSV-1 or HSV-2 isolates, has been reported to be even more sensitive and specific than the enzyme immunoassays (Ashley et al., 1988, 1998), and is therefore considered as "the gold standard" for the diagnosis of HSV-1 and HSV-2 specific serology.

The commercial assays have some limitations. The cost is a problem for routine usage in developing countries. The reliability of methods using the complete glycoprotein G as antigen has been questioned recently since the serostatus of some individuals with definite HSV-2 infection changed from being HSV-2 antibody positive to HSV-2 antibody negative (Schmid et al., 1999). Alternative methods have been described. Liljeqvist et al. (2002) have reported recently that the secreted portion of gG-2 reacts specifically with HSV-2 antibodies. It was shown previously that a low-cost synthetic oligopeptide corresponding to a region of gG-2 performed slightly better than commercial enzyme immunoassay for detection of HSV-2 antibodies (Oladepo et al., 2000; Nilsen et al., 2003). Since an HSV-1 specific epitope has been identified recently on gG-1 (Tunbäck et al., 2000), we have explored the possibility of establishing a peptide-based assay for specific detection of HSV-1 antibodies. The results presented in this paper show that the method performs as well as the commercial ones. We suggest that this inexpensive, reliable and rapid assay for HSV-1 antibodies could facilitate routine high through put serodiagnosis of HSV-1 infections.

2. Materials and methods

2.1. Sera

A total of 112 human sera were examined, 64 from Africa and 48 from Scandinavia. African sera were from Tanzania and the Scandinavian ones from Norway and Sweden. Since performance of Focus commercial assays in children under 14 years is not known (Ashley, 2001), the 112 sera used were from patients which were at least 15 years old.

All sera were analysed for the presence of HSV-1 and HSV-2 antibodies by two commercial assays, HerpeSelect 1 and 2 Immunoblot IgG (IB0900G) and HerpeSelect 1 enzyme-linked immunosorbent assay (ELISA) IgG (EL0910G) from Focus Technologies Inc. (USA). According to the manufacturer the sensitivity and specificity of the immunoblot strips relative to Western blot are 99 and 92%, respectively for HSV-1, while those for HSV-2 are 98 and 94%. In comparison to Western blot the sensitivity and specificity of the HerpeSelect 1 ELISA are 98 and 94%, respectively (Ribes et al., 2002). Four groups of sera were obtained, as shown in Table 1. Group 1 sera were HSV-1 positive and HSV-2 negative, group 2 was negative for both HSV-1 and HSV-2 antibodies, group 3 was HSV-1 negative and HSV-2 positive, while group 4 was both HSV-1 and HSV-2 positive.

2.2. Synthesis of oligopeptides

The HSV-1-specific immunodominant region identified in gG-1 is delimited by amino acids 112–127 (Tunbäck et al., 2000). Three branched peptides, each containing a four-glycine spacer between the epitope and the lysine core, spanning this region were synthesized by continuous N'-9-fluorenylmethyloxycarbonyl (FMOC) chemistry as

Table 1
Groups of sera characterized by a commercial western blot assay and by a commercial ELISA method

Origin of sera		Group 1 (HSV-1 positive/HSV-2 negative)	Group 2 (HSV-1 negative/HSV-2 negative)	Group 3 (HSV-1 negative/HSV-2 positive)	Group 4 (HSV-1 positive/HSV-2 positive)
S[a]	48	17	7	16	8
A[b]	64	35	12	0	17
Total	112	52	19	16	25

[a] Scandinavian.
[b] African.

described previously (Marsden et al., 1998). Peptide 2048.8 of structure $(PLAEDVEKDKPNRPVVGGGG)_4K_3A$ corresponded to the 16 amino acid sequence of the epitope. Peptide 2048.9 $(PAFPLAEDVEKDKPNRPVVGGGG)_4K_3A$ contained the three additional amino acids, PAF, flanking the N-terminus of the epitope. Peptide 2048.10 $(PLAEDVEKDKPNRPVVPSPGGGG)_4K_3A$ contained the three additional amino acids PSP flanking the C-terminus of the epitope. Four copies of each peptide were synthesized onto a branched lysine core (Tam, 1988).

2.3. Peptides in enzyme-linked immunosorbent assay (ELISA)

The peptides were dissolved in water and diluted further in phosphate-buffered saline (PBS) to the concentrations indicated in the text. ELISA was carried out as described previously (Marsden et al., 1998). Briefly, 50 μl of peptide solution was added to each well in a microtiter plate (Immunlon 1B, 96 wells, Thermo Labsystems, USA) and the peptide was allowed to adsorb to the plate at 4 °C overnight. Various amount of peptide were used, as described in Section 3. The antigen solution was then removed and unoccupied unspecific binding sites on the plate blocked by incubating for 1.5 h at 37 °C with PBS containing 1% bovine serum albumin (BSA). Wells were washed six times with 150 mM NaCl containing 0.05% Tween 20 before addition of 50 μl diluted serum and incubation for 1.5 h at room temperature in an orbital shaker. Washing was repeated as above prior to addition of 50 μl biotinylated sheep anti-human IgG (Amersham) diluted 1/1000 and incubation performed for 1.5 h at 37 °C. Followed by another washing, 50 μl of streptavidin-conjugated horseradish peroxidase (Amersham) diluted 1/1000 was added and incubation repeated at 37 °C as above before washing. The colour was developed by incubating at room temperature in citrate buffer containing *o*-phenylenediamine dihydrochloride (OPDA) and hydrogen peroxide, and the reaction stopped by addition of 50 μl 2N sulfuric acid. The absorbances were read at 492 nm in a Labsystem Multiscan MS plate reader.

3. Results

3.1. Performance of the three different oligopeptides in the ELISA

The three oligopeptides differed by the presence or absence of flanking amino acids corresponding to those in gG-1, as described in Section 2. Initially we tested whether these additional amino acids had any influence on the performance of the peptides by ELISA. A panel of 31 characterized Scandinavian sera was analysed. Seventeen sera were HSV-1 positive/HSV-2 negative (group 1), seven were negative for both HSV-1 and HSV-2 (group 2) and seven HSV-1 negative and HSV-2 positive (group 3). The sera were diluted five-fold (1/5) and 1.0 μg peptide used per well.

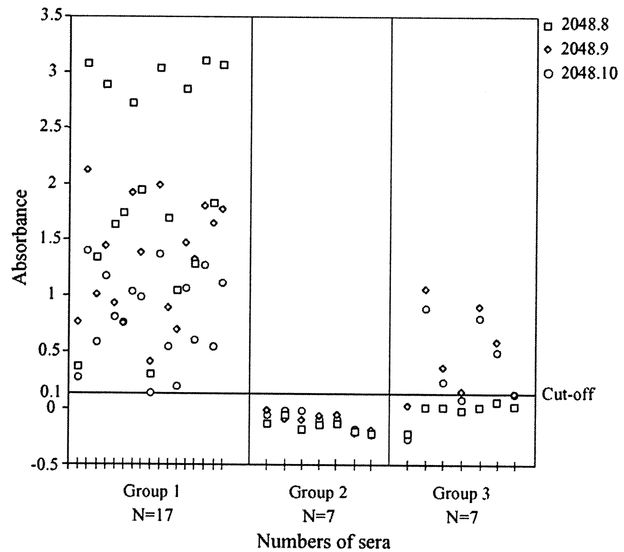

Fig. 1. Initial analysis of the performance of three different gG-1 oligopeptides in an ELISA. The peptides and the ELISA in which they were used are described in Section 2. Three different groups of sera were analysed. Group 1 shows the reactivities of HSV-1 positive and HSV-2 negative sera, group 2 shows the reactivities of HSV-1 negative/HSV-2 negative sera and group 3 shows reactivities of HSV-1 negative/HSV-2 positive sera. The cut-off was chosen as described in Section 3.

The absorbance values of each serum with control (PBS-coated) wells was subtracted from the absorbance values with peptide-coated wells and a cut-off value of 0.1 was chosen. The results are shown in Fig. 1. For group 1 sera, peptide 2048.8 (without flanking a.a.) gave the highest absorbance in 15 of 17 cases, and peptide 2048.9 in the remaining two cases (specimens 1 and 9). When testing HSV-1 and HSV-2 negative sera, the lowest absorbances were obtained with peptide 2048.8 in five of seven cases. Possible crossreactivity with HSV-2 antibodies was tested by analysis of sera which were HSV-1 negative and HSV-2 positive (group 3). Peptide 2048.8 did not react while the other two peptides each reacted to various degrees in several cases. Based upon these results peptide 2048.8 was selected for further testing.

3.2. Optimization of the amount of peptide and sera in the ELISA

The wells of the microtiter plates were coated with five different amounts of peptide 2048.8, ranging from 5 μg to 5 ng. Three sera with HSV-1 antibodies only (group 1) were analysed at a series of two-fold dilution. These sera gave high, medium and low absorbances, respectively, in the initial testing. The results obtained with the high absorbance and low absorbance sera are shown in Fig. 2. For the highly reactive serum (Fig. 2A) the absorbance was markedly above background at all amounts of peptide, and at a serum dilution of at least 1/40. The serum with low reactivity in the initial test, however, gave lower absorbances at the higher than at

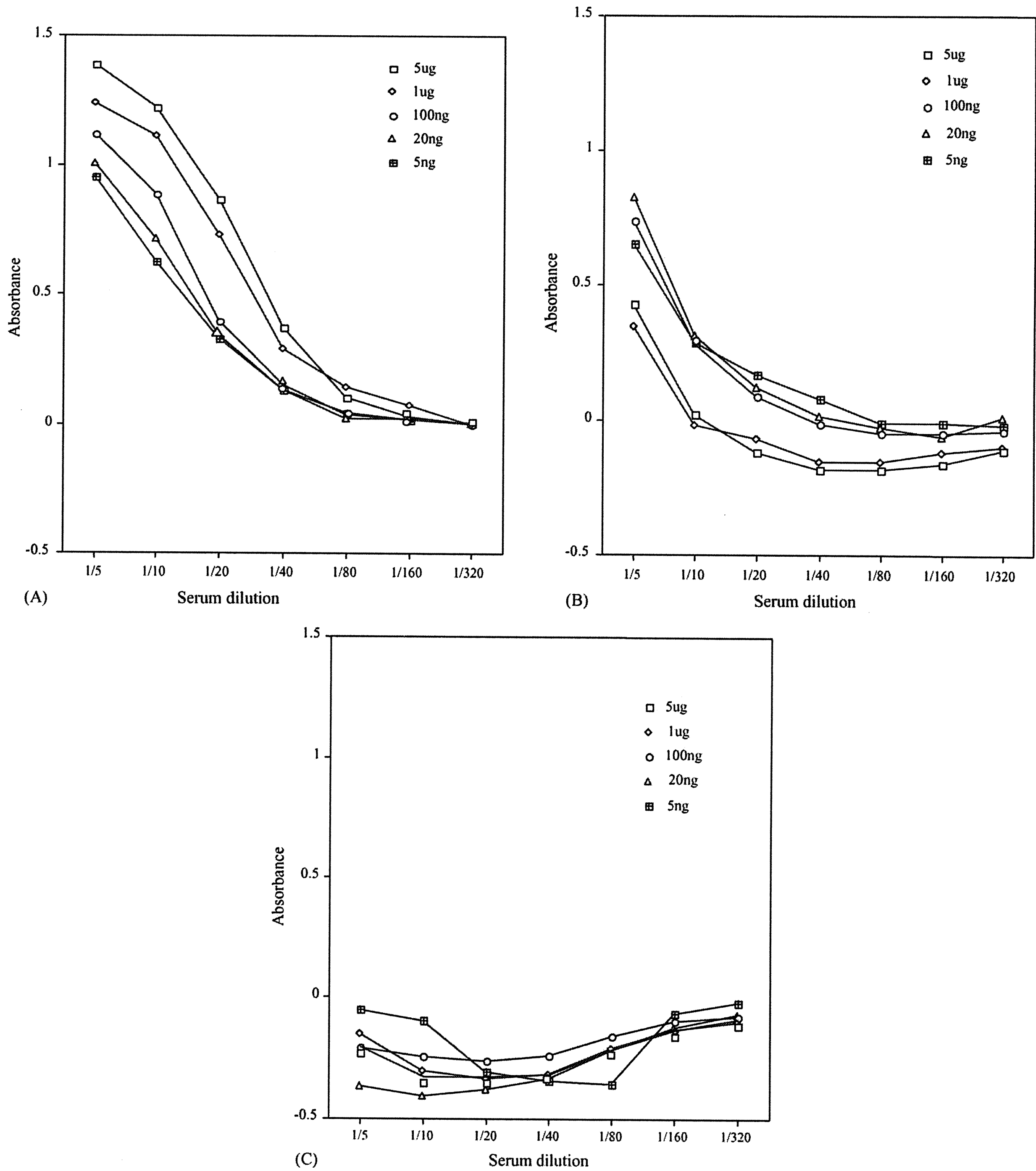

Fig. 2. Optimization of the amount of peptide 2048.8 and serum to be used in the ELISA method. Ninety-six well ELISA plates were coated with different amounts of peptide 2048.8, ranging from 5 μg to 5 ng. Sera were analysed at series of two-fold dilution. Two HSV-1 positive/HSV-2 negative sera, one with high and the other with low absorbance are shown in panels A and B. Panel C shows the results from a HSV negative serum.

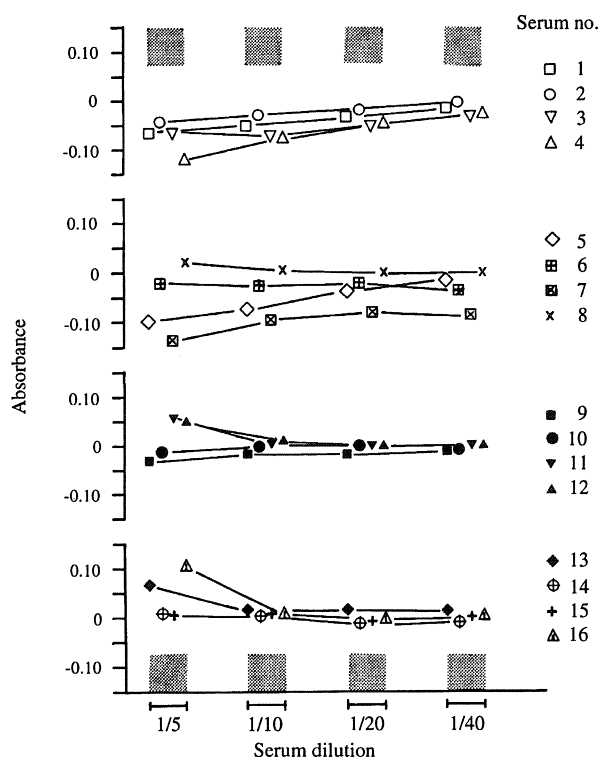

Fig. 3. Determination of the optimal serum dilution to avoid crossreaction with HSV-2 antibodies. Sixteen HSV-1 negative/HSV-2 positive sera were analysed. The wells were coated with 100 ng of peptide 2048.8 and the sera were diluted 1/5, 1/10, 1/20 and 1/40.

the lower amounts of peptide (Fig. 2B) at all dilution of the serum. When using peptide in the range 5–100 ng per well the absorbance was markedly above background at a serum dilution 1/10. A serum from the group lacking both HSV-1 and HSV-2 antibodies (group 2) was tested under the same conditions, and the results are shown in Fig. 2C. The absorbances were below background under all experimental conditions. Taken together these results indicated that 100 ng of peptide per well and a serum dilution of 1/10 would be appropriate in further testing.

The optimal serum dilution should give as high absorbance as possible under conditions where there is no cross reaction. The 16 sera in the group HSV-1 negative/HSV-2 positive were therefore analysed at various dilutions, using 100 ng peptide per well. The results are shown in Fig. 3. At a dilution of 1/5 five sera (specimens 8, 11, 12, 13 and 16) had an absorbance slightly above background, but there was only background at dilutions 1/10 and higher. The sera should consequently be diluted 1/10 to avoid cross-reaction.

3.3. Optimization of other reagents and steps in the ELISA

To ensure that the second antibody and hydrogen peroxide were not limiting in the ELISA reactions, assays were performed at various concentrations of these reagents. The results (data not shown) demonstrated that there was surplus of second antibody at a dilution of 1/1000, and of H_2O_2 at a concentration of 0.03% in the citrate buffer containing the chromogenic substrate.

The time course for colour development was then determined under the optimized conditions, using one serum with a high and another one with a low absorbance in the previous experiments. The reaction was completed within 15 min in both cases (data not shown).

3.4. The final recipe

After adjusting the method accordingly, peptide 2048.8 was chosen at a concentration of 100 ng per well, a serum dilution of 1/10 and H_2O_2 at a concentration of 0.03%. The colour should be allowed to develop for at least 15 min.

3.5. Reproducibility of the assay

Five different sera were analysed to determine the intra-assay and inter-assay variations, respectively. One serum did not contain HSV-1 or HSV-2 antibodies. The four other sera were either HSV-1 positive/HSV-2 negative or HSV-1 negative/HSV-2 positive, one with a high and the other one with a low absorbance. The HSV-1 negative/HSV-2 positive sera were all below cut-off, but among these the sera with the highest and lowest absorbance were chosen. The inter-assay variation was determined by analysing each serum mentioned above in four consecutive runs, and the intra-assay variation was determined by analysing six parallels of each serum mentioned above in one test run. The results are shown in Tables 2 and 3. The mean inter-assay and intra-assay variations were 8.1 and 8.8%, respectively.

Table 2
Inter-assay variation of the peptide ELISA method

Sample	Absorbance at different days of testing			
	Day 1	Day 2	Day 3	Day 4
1. HSV-1 positive/HSV-2 negative (high absorbance)	2.493	2.932	2.490	2.586
2. HSV-1 positive/HSV-2 negative (low absorbance)	0.346	0.316	0.227	0.320
3. HSV-1 negative/HSV-2 negative	0.060	0.066	0.064	0.060
4. HSV-1 negative/HSV-2 positive	0.067	0.072	0.064	0.069
5. HSV-1 negative/HSV-2 positive	0.056	0.049	0.054	0.058

Coefficient of variation is 8.1%.

Table 3
Intra-assay variation of the peptide ELISA method

	Absorbance in parallel experiments					
	1	2	3	4	5	6
1. HSV-1 positive/HSV-2 negative (high absorbance)	2.586	2.871	2.854	2.777	2.511	2.775
2. HSV-1 positive/HSV-2 negative (low absorbance)	0.262	0.273	0.350	0.301	0.271	0.231
3. HSV-1 negative/HSV-2 negative	0.060	0.061	0.064	0.064	0.063	0.066
4. HSV-1 negative/HSV-2 positive	0.069	0.071	0.080	0.085	0.071	0.075
5. HSV-1 negative/HSV-2 positive	0.042	0.040	0.049	0.039	0.043	0.046

Coefficient of variation is 8.8%.

3.6. *Performance of the assay in comparison with a commercial ELISA method*

A total of 112 sera from Scandinavia and Tanzania were characterized by the HerpeSelect Immunoblot method and by the HerpeSelect 1 ELISA IgG tests (31 of them were already tested, Fig. 1). All sera were then tested by the peptide ELISA, and for both Tanzanian and Scandinavian sera the results were the same as those obtained by the commercial tests (data not shown).

4. Discussion

A new, peptide-based ELISA method is described for detection of HSV-1 antibodies in human serum. An immunodominant region of 16 amino acids was previously identified in gG-1 by the reactivity of purified human anti-gG-1 antibodies to a number of different synthetic oligopeptides bound to a cellulose matrix (Tunbäck et al., 2000). The antigen in the present method is a branched synthetic oligopeptide containing the HSV-1 immunodominant region in gG-1. Since the patterns and amounts of immunoglobulins in human might be different in various parts of the world, sera were collected both from Scandinavia and Africa and characterized as described in Section 2. When analysing all sera with the peptide ELISA, a commercial ELISA kit and a commercial immunoblot method, the results were identical, showing that the sensitivity and the specificity of the new method are the same as of the commercial kits.

Commercial methods for specific and sensitive detection of HSV-1 or HSV-2 antibodies are based upon the antigenicities of the complete glycoproteins gG-1 and gG-2 in immunoblot or ELISA methods. It has been demonstrated, however, that the glycoprotein G-1 gene is present in a number of variants in HSV-1 isolated from a Scandinavian population (Rekabdar et al., 1999). This variability, which might be even higher among African isolates considering the population history, could possibly influence the IgG response to gG-1 in the host (Rekabdar et al., 2002). The results obtained with the peptide-ELISA, however, were identical to those obtained with the immunoblot assay of both Scandinavian and African sera.

Initially, three different oligopeptides were tested. The results were consistent with those of the immunoblot method only when the peptide corresponded in sequence to the HSV-1 immunodominant region. Addition of three flanking amino acids, corresponding to those in gG-1, to either the N-terminal or the C-terminal end of this peptide reduced both the specificity and the sensitivity. Consequently, we did not use these peptides.

Since only a few persons are expected to acquire HSV-2 infection without having had a previous HSV-1 infection, it is rather difficult to obtain sera in the group HSV-1 negative/HSV-2 positive. We were able to collect some Scandinavian sera in this group, but unfortunately none from Africa in spite of analysis of several hundred sera. Sera in this group are valuable for testing cross-reactivity between HSV-2 antibodies and the putative HSV-1 specific antigen. When carrying out the peptide ELISA method at a serum dilution of 1/10 and with 100 ng antigen per well, there was no detectable cross-reaction, but low reactive HSV-1 positive sera did still show absorbances significantly above background.

In conclusion, the present method for detection of HSV-1 antibodies in human sera is sensitive, specific, reliable and easy to use. It is an inexpensive, affordable method which can be used when the economical resources are limited.

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