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Detection of the four major human herpesviruses simultaneously in whole blood and cerebrospinal fluid samples by the fluorescence polarization assay

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SUMMARY

Objectives: Herpes simplex virus type 1/2 (HSV-1/-2), cytomegalovirus (CMV), and Epstein–Barr virus (EBV) correlate strongly with infections of the central nervous system. The objective of this study was to develop a method for the simultaneous detection of HSV-1/-2, CMV, and EBV DNA by the fluorescence polarization assay based on asymmetric polymerase chain reaction (PCR) and hybridization.
Methods: DNA of HSV-1/-2, CMV, and EBV was amplified in an asymmetric PCR by a universal primer system. The amplicons were then detected by the fluorescence polarization assay. In this method, the probes for HSV-1/-2, CMV, and EBV hybridized with their respective target amplicons, and the hybridization resulted in an increase in the fluorescence polarization values. Infections of HSV-1/-2, CMV, and EBV were determined by the increased fluorescence polarization values. The DNA extracted from whole blood and cerebrospinal fluid samples was subjected to fluorescence polarization and a previously published multiplex PCR assay in parallel.
Results: Compared to the multiplex PCR assay, no significant difference in the numbers of samples positive for the human herpesviruses was identified by the fluorescence polarization assay.
Conclusions: The fluorescence polarization assay presented in this study is a reliable, convenient, and cost-effective diagnostic tool that allows the detection of the four major human herpesviruses.
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1. Introduction

Infections of the central nervous system are a difficult diagnostic problem for both clinicians and microbiologists. Various clinical signs may be associated with infections caused by the four major human herpesviruses (HHVs): herpes simplex virus type 1/2 (HSV-1/-2), human herpesvirus type 4 (cytomegalovirus, CMV), and human herpesvirus type 5 (Epstein–Barr virus, EBV).[1] Identification of the DNA of the four major human herpesviruses in whole blood and cerebrospinal fluid (CSF) samples has become the most direct and reliable method for monitoring HHV infections. However, conventional detection methods for HSV-1/-2, CMV, and EBV are not used in routine practice due to the complex experimental process and high costs.[2–6]

A few fluorescence polarization (FP) assays have been used as the diagnostic method for the detection of particular organisms.[7,8] The FP assay is simple as it can directly detect the hybridization between the fluorescent-labeled probes and the complementary single-stranded DNA in polymerase chain reaction (PCR) products.[9] In this study we developed an FP assay for the simultaneous detection of the four major human herpesviruses in whole blood and CSF samples. Asymmetric PCR mainly generates single-stranded DNA, which is more suitable for the hybridization–FP assay than double-stranded DNA generated by symmetric PCR;[10] hence single-stranded DNA was used in the current study.

2. Materials and methods

2.1. Primers and probes

A set of universal primers (H-GF and H-GR) within a highly conserved region of the DNA polymerase gene from the four herpesviruses was designed by Rozenberg and Lebon.[11] The primers span a polymorphic inner region and were used to amplify the fragments of the DNA polymerase gene from HSV-1/-2, EBV, and CMV. The four probes hybridized with one of the respective strands of the amplicons of HSV-1/-2, EBV, and CMV were designed within the target amplification polymorphism regions of DNA polymerase

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Table 1
Primers and probes labeled

Genotype	Sequence (5′→3′)	Fluorescence labeled
H-GF	gactttgccagcctgtacc	
H-GR	gtccgtgtccccgtagatg	
HSV1-Probe	caaggctcacgtgcgagagagcctcctc	-FAM
HSV2-Probe	gaaggcccacgtacgcgagagcctgctga	-TAMRA
CMV-Probe	gtatacagcgtcacgctagagaacggcgt	-ROX
EBV-Probe	cacgaaggccttggcccgctccaacatc	-BTR

HSV, herpes simplex virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus.

genes for the corresponding herpesvirus on the basis of sequence information derived from NIH-GenBank. A database search (BLAST from National Center for Biotechnology Information) of the probe sequences revealed complete matches with their corresponding herpesviruses. The four probes were labeled respectively with TAMRA (carboxytetramethylrhodamine), FAM (carboxyfluorescein), ROX (carboxy-X-rhodamine), and BTR (BODIPY-Texas Red) to differentiate the amplification of HSV-1/-2, EBV, and CMV. All the primers and probes were synthesized and labeled by Invitrogen (Shanghai, China) (Table 1).

2.2. Controls, samples, and DNA extraction

The following prototype strains were used in this research: CMV-AD169 (ATCC VR-538), EBV (ATCC VRL-1612), and HSV-1/-2 (PG21). HSV-1/-2 was cultivated in Vero cells, CMV was cultivated in MRC5 diploid cells, and EBV was obtained from B95-8 cells. The DNA isolated from the cells was amplified using H-GF and H-GR. The 518-bp DNA fragments of the DNA polymerase gene of HSV-1/-2, 524-bp fragments of the DNA polymerase gene of EBV, and 589-bp fragments of the DNA polymerase gene of CMV were cloned into pGEM-T-easy vectors to construct recombinant plasmids (pGEM-T-HSV-1, pGEM-T-HSV-2, pGEM-T-EBV, and pGEM-T-CMV) following the manufacturer's instructions (Promega, USA). The recombinant plasmids were identified by sequence. After purification of the plasmids using a Wizard DNA Miniprep Kit (Promega, USA), the amount of DNA was quantified by measuring the optical density at 260 nm, and the copy number was calculated. The plasmids were serially diluted with HHV-negative plasma from a concentration of 1.0×10^{10} copies to 1.0 copy. The prepared plasmids and their mixtures were used as positive controls. The prepared pGEM-T-easy vector plasmids were used as negative controls.

Genetically related virus DNA (human herpesvirus 6 and varicella-zoster virus) isolated from infected cells and unrelated virus DNA (plasmids of human papillomavirus and hepatitis B virus) were used in the specificity analysis.

Whole blood samples from 350 screened blood donors (Blood Bank, Shanxi, PLA), for which serological determination had been performed by enzyme-linked immunosorbent assay (ELISA; IBL International, Germany), were used as the control group. Whole blood samples from 832 patients with clinically suspected encephalitis or meningitis attending the Department of Neurology of Xijing Hospital and the Department of Pediatrics of Tangdu Hospital of the Fourth Military Medical University formed the patient group. Three hundred and twenty-two CSF samples were also collected from these patients.

EDTA peripheral blood and CSF samples were obtained from the patients within 24 h of hospitalization, before antiviral therapy. Total DNA was extracted from 200 μl of whole blood and CSF using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in 60 μl of TE buffer (10 mM Tris–HCl (pH 8.0) and 1 mM EDTA disodium salt); samples were stored at −70 °C.

All the samples were checked by PCR assay using primers specific for α-tubulin to exclude the presence of inhibitors and to assess the quality and the integrity of the extracted DNA from whole blood or CSF, as described previously.[3] Ten of the 322 CSF samples unamplified by α-tubulin primers were excluded from the study.

2.3. DNA amplification

All the positive and negative controls were subjected to asymmetric PCR. An asymmetric PCR was carried out in 25 μl PCR reaction buffer containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 5% dimethylsulfoxide, 5 μl template DNA, 2.5 U Taq polymerase (Promega, USA), 0.2 mM deoxynucleoside triphosphates (dNTP, each), and unequal amounts of the two primers (10 pmol H-GF and 50 pmol H-GR primers). The reaction mixture was incubated at 94 °C for 5 min. Next, it was subjected to 45 cycles of incubation at 94 °C for 60 s to denature the DNA, at 58 °C for 60 s to anneal the primers, and at 72 °C for 40 s to extend the annealed primers. Finally, it was subjected to 94 °C for 60 s, 58 °C for 60 s, and cooled to 25 °C.

2.4. Simultaneous detection of the four major human herpesviruses by the FP assay

First, the asymmetric PCR products of the positive and negative controls were detected by the FP assay with labeled probes, as previously described, with some modifications.[7] In brief, the probes were diluted to 2.5 nM with TE buffer containing 0.8 M NaCl. The positive hybridization contained a 200 μl mixture of the four probes (50 μl, each) and 25 μl of the asymmetric PCR products generated from the positive controls. The negative hybridization contained a 200 μl mixture of the four probes and 25 μl of the asymmetric PCR products generated from the negative controls. The mixes were incubated at 48 °C for 30 min and cooled to 25 °C. Reactions were performed in duplicate. The FP values of the reaction buffer were measured using the Fluorescence Polarization Capable Instrument (YG-06, Yangguang, Shanxi, China), as previously described.[7]

Next, the DNA extracted from each sample with successful amplification of α-tubulin was subjected to asymmetric PCR and the hybridization–FP assay. Each sample was analyzed in duplicate.

2.5. Detection of the four major human herpesviruses by the multiplex PCR assay

To confirm the accuracy of the FP assay, the DNA extracted from each sample with successful amplification of α-tubulin was subjected to the multiplex PCR assay as previously described.[3] The positive samples that gave rise to amplicons of the predicted size were excised and purified from the agarose gel using the DNA Midi Purification Kit (Tiangel, Beijing, China). The detection of the four major human herpesviruses was achieved by digestion with the restriction enzymes *HpaII* and *Tru9I* (Promega, USA).

3. Results

3.1. Cut-off values and the four major human herpesviruses in samples

The average FP values (means) and the standard deviations (SD) of the negative/positive controls for HSV-1/-2, CMV, and EBV are shown in Table 2. The average FP values of the positive controls were compared with the average FP values of the negative controls. The average FP values (means) of the negative and the positive data sets for HSV-1/-2, CMV, and EBV were significantly different at a 99% level of confidence in *t*-distribution tests. According to the statistical certification test (*t*-test), the cut-off values for each target were assured at a confidence level of >99%.[7,12]

Table 2
Fluorescence polarization values (units are millipolarization, mP) of controls and samples for herpesvirus types

Herpesvirus type	FAM	TAMRA	ROX	BTR
Negative controls (1.0–1.0 × 10^{10} copies)				
pGEM-T-easy	34.42 ± 1.7	46.79 ± 1.9	57.23 ± 2.5	36.23 ± 1.9
Positive controls (1.0–1.0 × 10^{10} copies)				
pGEM-T-HSV-1	**126.51 ± 4.5**	55.86 ± 3.94	64.87 ± 4.98	43.33 ± 2.7
pGEM-T-HSV-2	38.4 ± 2.7	**163.339 ± 5.6**	68.74 ± 4.94	48.21 ± 3.5
pGEM-T-CMV	41.55 ± 3.2	60.78 ± 4.66	**193.89 ± 6.1**	45.58 ± 3.7
pGEM-T-EBV	40.9 ± 3.5	57.66 ± 4.67	65.37 ± 4.7	**143.76 ± 4.9**
Mixture of four plasmids	**128.17 ± 4.9**	**159.44 ± 4.9**	**190.06 ± 5.8**	**136.32 ± 5.1**
Whole blood (1182)				
HSV1 (63)	**137 ± 4.8**	58.6 ± 3.8	63.79 ± 4.7	45.13 ± 3.8
HSV2 (43)	39.7 ± 3.9	**164.9 ± 5.6**	69.84 ± 5.2	49.88 ± 4.3
CMV (145)	45.34 ± 3.7	61.89 ± 5.6	**196.3 ± 5.1**	43.43 ± 3.8
EBV (75)	37.4 ± 3.6	58.87 ± 4.7	66.28 ± 4.9	**154.55 ± 6.4**
Herpesvirus-negative (874)	41.77 ± 2.9	54.35 ± 2.6	62.66 ± 4.4	46.82 ± 2.7
CSF (312)				
HSV1 (17)	**144.5 ± 4.7**	60.4 ± 3.4	65.7 ± 4.9	48 ± 4.9
HSV2 (11)	41.2 ± 3.4	**171.9 ± 5.8**	64.9 ± 5.7	52.6 ± 4.7
CMV (53)	44.4 ± 3.6	63.3 ± 5.4	**201.6 ± 5.7**	53.4 ± 4.6
EBV (13)	39.4 ± 4.2	62.8 ± 4.9	59.5 ± 4.2	**171.7 ± 5.9**
Herpesvirus-negative (224)	45.4 ± 3.7	59.4 ± 4.5	68.4 ± 5.3	56.2 ± 4.1

HSV, herpes simplex virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus.
The fluorescence polarization (FP) values are represented as mean ± standard deviation. The FP values units are millipolarization (mP). Results are stratied by herpesvirus types and fluorophores.

The DNA extracted from the whole blood and the CSF samples was analyzed for the presence of HSV-1/-2, CMV, and EBV by the FP assay. The average FP values of the sample were compared with the average FP values of the negative controls. If the net change in the FP value of a sample for FAM was >38 mP (units of millipolarization) of the negative control, the sample was identified as positive for HSV-1. If the net change in the FP value of a sample for TAMRA was >38 mP of the negative control, the sample was identified as positive for HSV-2. If the net change in the FP value of a sample for ROX was >42 mP of the negative control, the sample was identified as positive for CMV. If the net change in the FP value of a sample for BTR was >42 mP of the negative control, the sample was identified as positive for EBV. If there was a great enough net change in the FP values for more than one fluorophore in a sample, the sample was identified as co-infected (Table 3). For the positive samples, the FP value ranges were 91–193 mP for HSV1, 114–242 mP for HSV2, 126–264 mP for CMV, and 96–237 mP for EBV. The indeterminate samples after the first analysis were re-analyzed using the same method. There were no equivocal results. The same infections were found in the positive CSF samples and their matched whole blood samples.

3.2. Analytical sensitivity

The sensitivity of the method was identified using serial diluted plasmids. Analysis was performed with 20 replicates per concentration level. The percent positivity was calculated for each target at every concentration level, particularly at the cut-off level. The minimum level at which at least 95% of the reactions were positive was 500 copies/ml for HSV-1/-2, EBV, and CMV, respectively. Compared with the multiplex PCR assay of Markoulatos et al. , a higher sensitivity was observed with the FP assay.[3]

The sensitivity of the method for the detection of co-infections was examined. HHV-negative plasma containing the plasmids pGEM-T-HSV-1, pGEM-T-HSV-2, pGEM-T-EBV, and pGEM-T-CMV were mixed in each of the following ratios: 25:25:25:25; 49:25:25:1; 25:49:1:25, and 1:25:25:49. The FP assay was able to detect the minor population of HHV type even when its content was as low as 1%.

A panel of positive samples with different DNA concentrations ranging from 1 to 500 ng was tested. The FP values were sufficiently different from the negative controls across the entire range of DNA template amounts.

3.3. Analytical specificity

The specificity of the primers was evaluated against unrelated viruses (plasmids of human papillomavirus and hepatitis B virus) and pGEM-T-easy vector in the presence of 10 μg of DNA extracted from the HHV-negative plasma. None of them was amplified. This suggests that the universal primers were highly specific and only the target gene could be amplified efficiently.

The four probes exhibited strong specificity for their corresponding herpesviruses. The assay was able to detect each viral standard plasmid from 5 to 1 × 10^{10} copies per reaction without cross-reaction in the presence of 1 μg of DNA extracted from the HHV-negative plasma.

Potential cross-reactivity was evaluated against other related virus DNA (human herpesvirus 6 and varicella-zoster virus) in the

Table 3
Number of samples for herpesvirus types identified by fluorescence polarization and multiplex PCR assay

Specimen	FP					Multiplex PCR				
	CMV	EBV	HSV1	HSV2	Co-infection	CMV	EBV	HSV1	HSV2	Co-infection
Blood										
Patient group (832)	142	75	61	43	17	142	75	60	42	16
Control group (350)	3	0	2	0	0	3	0	2	0	0
CSF										
Patient group (312)	53	13	17	11	5	53	13	16	11	5

FP, fluorescence polarization; CMV, cytomegalovirus; EBV, Epstein–Barr virus; HSV, herpes simplex virus.
Co-infection means that the sample was infected simultaneously with more than one of the four major human herpesviruses.

presence of 1 μg of the DNA extracted from the HHV-negative plasma. No cross-reaction was observed.

Clinical specificity was evaluated by analysis of 350 samples from screened blood donors that were non-reactive for HHV by immunoassay. CMV DNA was detected in three and HSV-1 DNA was detected in two of the samples. Confirmation of the results was achieved by multiplex PCR assay.

3.4. Analytical stability

The serial diluted plasmids (5–1 $\times$ 10^{10} copies) of the positive/negative controls were observed within run, run-to-run in three nonadjacent laboratories. Analysis was performed with 10 replicates per concentration level. Twenty-two repeat runs of the 10 replicates were performed at each site. The average FP values were calculated at each concentration level. The assay of the FP values indicated that there was excellent reproducibility in the method.

Reproducibility was evaluated using DNA extracted from 200 positive samples from the patient group and 100 negative samples from the control group within run, run-to-run in three separate, nonadjacent laboratories. The infection in each sample was the same.

3.5. Comparison of the FP assay with the multiplex PCR assay

All samples were subjected to the FP and multiplex PCR assay in parallel. The detection of HSV-1/-2, EBV, and CMV infection based on the FP and the multiplex PCR assay was compared by Chi-square test using SPSS v.10.0 software (SPSS Inc., Chicago, IL, USA). No significant difference in the numbers of samples positive for the HHVs was identified between the two assays. Compared with the multiplex assay, the sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) of the FP assay for the blood specimens of the two groups were 95%, 98%, 95%, and 98%, respectively; the sensitivity, specificity, NPV, and PPV for the CSF specimens were 92%, 96%, 91%, and 96, respectively.

The discordant results for herpesvirus types between the two assays were confirmed using the nested PCR assay as described previously.[13]

4. Discussion

We have described the detection of the four major human herpesviruses in whole blood and CSF samples by the FP assay. A comparison between the FP assay and the multiplex PCR assay confirmed the accuracy of the method in a large number of samples. Of the blood samples in the control group non-reactive for HHV by ELISA, three tested positive by the FP assay for CMV DNA and two tested positive by the FP assay for HSV-1. This demonstrates that the FP assay was more sensitive than the ELISA for the detection of HSV and CMV,[14] so it is more appropriate for screening of samples that are non-reactive by immunoassay.

For the FP assay, the threshold concentration was dependent on the target gene concentration. Sufficient products were required for the probes to bind entirely. There would be a lower net change in a sample in which the probes are partially bound than in a sample in which the probes are entirely bound. If only a small number of amplicons was formed in a sample with a low concentration of target gene, giving low FP values, such a sample would be identified as negative.

The FP assay is reliant on the hybridization of label probe with one strand of denatured PCR product. There are two competing equilibria in the reaction. These are the binding of the probe with one of the amplicon strands and the re-annealing of the two amplicon strands. More re-annealing of the two amplicon strands leaves more probe unbound. In our asymmetric PCR, a primer ratio of 1:5 and a rather high number of 45 cycles were used to ensure enough single-stranded product and the high sensitivity of the FP assay. The sensitivity of the assay makes it feasible for use in the detection of latent multiplex herpesviruses, which correlate closely with encephalitis and meningitis in infants and children. The FP assay could help to guide the initiation of appropriate antiviral therapy.

The FP assay can be completed in less than 4 h as it requires no separation, therefore the assay has the potential for real-time clinical applicability. The FP assay uses much lower quantities of reagents because the four types can be amplified by a single PCR and the PCR product can be detected in a single tube. Such a method is advantageous for the detection of viral encephalitis agents, especially where small sample volumes make the performance of multiple assays difficult.

Several methods, such as the multiplex real-time PCR assay, are now the preferred methodology. The minimum detection level of the multiplex real-time PCR assay is 2 copies per reaction for EBV, CMV, and HHV-6.[15] Compared with the multiplex real-time PCR method, a slightly lower sensitivity was observed with the FP assay. The advantage of the FP assay is that it is cost-effective as it does not require the instrument for real-time monitoring of amplification and the TaqMan probe; the cost of the probe used in the FP assay is only 20% of that of a dual-labeled TaqMan probe.

Dual or triple infections of the central nervous system by two or three herpesviruses have been reported.[16] There was no discordance between blood and CSF samples in this research. The feasibility of doing a single CSF analysis given the low incidence of herpesvirus encephalitis will be studied in a larger number of CSF samples.

The FP assay presented in this study could be a reliable, convenient, and cost-effective diagnostic tool. However, this test in its current form will have most utility in the initial detection of the four major human herpesvirus, and a lesser role in monitoring diseases, which is currently often done by quantitative PCR. The application of the FP assay in quantitative PCR will be studied in the future.

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