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Original Article

Development and evaluation of the quantitative real-time PCR assay in detection and typing of herpes simplex virus in swab specimens from patients with genital herpes

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Abstract: Genital herpes (GH), which is caused mainly by herpes simplex virus (HSV)-2 and HSV-1, remains a worldwide problem. Laboratory confirmation of GH is important, particularly as there are other conditions which present similarly to GH, while atypical presentations of GH also occur. Currently, virus culture is the classical method for diagnosis of GH, but it is time consuming and with low sensitivity. A major advance for diagnosis of GH is to use Real-time polymerase chain reaction (PCR). In this study, to evaluate the significance of the real-time PCR method in diagnosis and typing of genital HSV, the primers and probes targeted at HSV-1 DNA polymerase gene and HSV-2 glycoprotein D gene fraction were designed and applied to amplify DNA from HSV-1 or HSV-2 by employing the real-time PCR technique. Then the PCR reaction system was optimized and evaluated. HSV in swab specimens from patients with genital herpes was detected by real-time PCR. The real-time PCR assay showed good specificity for detection and typing of HSV, with good linear range (5×10^2~5×10^8 copies/ml, r=0.997), a sensitivity of 5×10^2 copies/ml, and good reproducibility (intra-assay coefficients of variation 2.29% and inter-assay coefficients of variation 4.76%). 186 swab specimens were tested for HSV by real-time PCR, and the positive rate was 23.7% (44/186). Among the 44 positive specimens, 8 (18.2%) were positive for HSV-1 with a viral load of 8.5546×10^6 copies/ml and 36 (81.2%) were positive for HSV-2 with a viral load of 1.9861×10^6 copies/ml. It is concluded that the real-time PCR is a specific, sensitive and rapid method for the detection and typing of HSV, which can be widely used in clinical diagnosis of GH.

Keywords: Genital herpes, herpes simplex virus, real-time polymerase chain reaction, detection, typing

Introduction

Genital herpes (GH) is a common chronic sexually transmitted infection worldwide with substantial morbidity caused mainly by herpes simplex virus type 2 (HSV-2) and sometimes by HSV-1 [1, 2]. The strongest known risk factor for the heterosexual transmission of human immunodeficiency virus (HIV) and other sexually transmitted infections is genital ulcer disease (GUD) [3]. Over the past decade, HSV has been identified as the most common etiological agent of GUD [4]. HSV seroprevalence increases with high risk sexual behavior and with factors related to polygynous marriage practices in rural populations [5, 6]. The majority of subjects infected with HSV are asymptomatic but exhibit sub-clinical cervicovaginal virus secretion which is thought to be important in the transmission of HSV [7].

Laboratory confirmation of GH is important, particularly as there are other conditions which present similarly to GH, while atypical presentations of GH also occur. The classical method for diagnosis and typing of HSV infections has been virus culture, but this method is time-consuming and has a low sensitivity, especially for the analysis of swab specimens [8]. More recently, polymerase chain reaction (PCR) methods have been actively investigated for the detection of HSV DNA in mucocutaneous lesions and have shown to be superior to viral culture [9]. A major advance for PCR diagnosis

of HSV infection is to use real-time PCR for detection and quantification. Amplification of the target DNA, and hybridization to the subtyping specific fluorescent probes are conducted in a single PCR and therefore the chances of possible contamination are minimized [10]. Real-time PCR has also been proved to be more sensitive in detecting asymptomatic shedding or shedding episodes in the absence of clinically obvious lesions [11]. In addition, for pathogenesis studies and clinical management purposes, including prognosis or determining optimal drug regimens, quantification of actual viral load by real-time PCR assay may be useful [7-11]. The present study aims to establish a quantitative real-time PCR method in the detection and typing of HSV and to evaluate the significance of this method in the diagnosis of GH.

Material and methods

Study subjects and specimens

In the present study, a total of 186 swab specimens from 186 patients with suspected genital lesions collected from July 2008 to April 2014 by the department of dermatology of the 306 Hospital of PLA were retrospectively investigated. The patients included 117 (58%) men and 69 (42%) women, with a median age of 36.3 (range 18-56). The patients' swab specimens were submitted to the clinical microbiology research center in 306 Hospital of PLA for diagnosis of HSV infection. In accordance with the inclusion criteria, patients selected include those with dirty sexual history and those whose spouses were diagnosed with HSV infection, with primary or recurrent clinical signs of tufted blisters, tiny fissures, erosion, ulcer, or with swelling in genital areas, but patients with genitourinary system diseases were not included. The initial samples were drawn from 0 to 6 days after the onset of clinical symptoms, before treatment. Specimens were collected directly from genital skin, the cervix, or the perianal region with Dacron swabs, and the swabs were placed into 1 ml of sterile normal saline solution. All samples were stored at -20°C until tested by both HSV culture and quantitative real-time PCR assay.

Virus strains

The international control SM_{44} strain of HSV-1 and Sav strain of HSV-2 were used as positive controls and titration determinations, and were obtained from the National Vaccine & Serum Institute. These viruses were grown and passaged according to standard virological methods [12]. Epstein-Barr virus (EBV), cytomegalovirus (CMV) and varicella-zoster virus (VZV) were provided by the Virus Institute of Chinese Preventive Medicine Academy of Science.

Primers, probes and target sequence for amplification

We designed separate primers and probes to distinguish between the two viral subtypes on the basis of the DNA polymerase and glycoprotein D gene of HSV [13, 14]. The HSV-1 typing primers and probe are directed to the HSV-1 DNA polymerase (UL42) gene fraction. The forward primer was 5'-GCCAGCGAGACGCTGAT-3', the reverse primer was 5'-ACGCAGGTACTCGTGGTGA-3', amplification fraction was 199 bp, and the fluorescent HSV-1 typing probe was 5'-CGCGAACTGACGAGCTTTGTGGT-3'. The HSV-2 typing primers and probe are directed to the HSV-2 glycoprotein D (US6) gene fraction. The forward primer was 5'-CACCACTTAAATCCTAAGGTTCC-3', the reverse primer was 5'-CTGATGACATAATTGAGATTGCACCC-3', amplification fraction was 124 bp, and the fluorescent HSV-2 typing probe was 5'-CAATTGCAGAAGACTTATTGCAC-3'. The probes was labeled at the 5' end with 6-carboxyfluorescein (FAM) and at the 3' end with 6-carboxytetramethylrhodamine (TAMRA).

Preparation of HSV DNA standards

The control SM_{44} strain of HSV-1 and Sav strain of HSV-2 were inoculated into single-layer cells to propagate. Pathological cells were collected when the number of cytopathic effects (CPE) cells reached 75%. DNA was extracted from pathological cells by phenol, chloroform and isoamylalcohol as template. The size of the PCR amplification products were 199 bp and 124 bp respectively from HSV-1 and HSV-2 of primers. This amplicon was further digested with BamHI/EcoRI, and cloned into the PUC18 plasmid (Invitrogen, Cergy Pontoise, France). The resulting PUC18-HSV plasmid was then purified with a QIAGEN plasmid Maxi kit (QIAGEN Ltd, Crawley, UK) according to the manufacturer's recommendations. The DNA concentration was assessed by spectrophotometry at 260 nm and

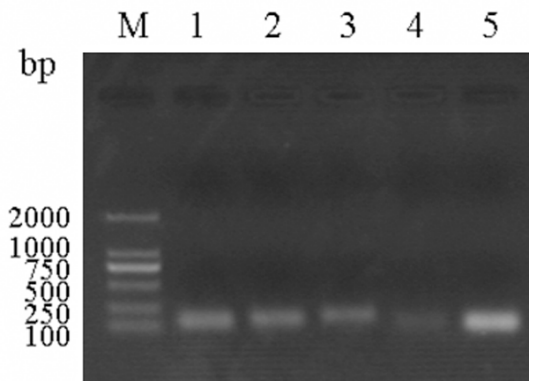

Figure 1. Gel electrophoresis of amplification product by real-time PCR. M: DL2000 DNA marker; 1, 5: HSV-2; 2-4: HSV-1.

was calculated as the average of three measurements. The plasmid was used in all experiments as a standard to quantify HSV DNA load.

Detection of HSV by real-time PCR

HSV DNA was detected and quantified in the acellular fraction of genital swab specimens by real-time PCR. The samples were thawed and left at room temperature, and were then centrifuged at 12,000× *g* for 5 min. The precipitate was dissolved into 50 µl of filter-sterilized lysis buffer (DA AN Gene company, Guangzhou city, China) and the solution was vortexed again and placed in a 70°C heat block for 10 min. The tubes were then placed in a 95°C heating block for 15 min to inactivate the proteinase, and centrifuged at 12,000× *g* for 5 min to elute the DNA. The final 30 µl PCR reaction mix contained 20 µl of 1× TaqMan universal master mix (PE Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, 0.2 µM of each primer and probe, and 2 µl of sample DNA. PCR was performed using a SLAN 96P Real-Time PCR System (Hongshitech, Shanghai, China) under the following cycling conditions: denaturation at 93°C for 2 min, 10 cycles of denaturation at 93°C for 45 s, annealing/extension at 55°C for 1 min, then followed by 30 cycles of 93°C for 30 s, 55°C for 45 s, and at 55°C with continuous fluorescence acquisition. Each PCR run contained several negative controls, including two reaction mixtures without DNA as well as several specimens that were known to contain no HSV DNA, a positive amplicon control, and a standard dilution curve for amplicon DNA. Each specimen was run in duplicate.

Virus isolation

Samples were inoculated into BHK_{21} cells in roller tubes, 0.2 ml per tube, and absorbed for 1 h at 37°C. The medium was replaced, and tubes were incubated at 37°C. Cultures were examined daily for CPE for 7 days for HSV [12]. HSV isolates were typed using D3 DFA reagent (Diagnostic Hybrids, Athens, OH).

Statistical analysis

HSV viral copy numbers were log transformed before statistical analysis. Statistical analysis was carried out in SPSS version 15 (SPSS Inc., Chicago, Illinois, USA). χ^2 tests were used for analysis of the data, and the significance level was set at $P<0.05$.

Results

Evaluation of the real-time PCR method

Specificity: By applying agarose electrophoresis to amplification product, the result showed that the size of specific sides were 199 bp and 124 bp from HSV-1 and HSV-2 control strains and PCR positive subjects (**Figure 1**). Fluorescence signal was presented in real-time PCR reaction well of HSV-1 and HSV-2 control strains, but not in those of EBV, CMV and VZV.

Linear range: Quantification was carried out by drawing standard curves using the serially diluted PUC18-HSV plasmid containing the target amplification product. Eight positive control standards at 10^1 to 10^8 copies/ml were used. The drawn HSV-1 standard curve was linear in 5×10^2 to 5×10^7 copies/ml, the linear regression coefficient was 0.997 and efficiency of PCR was 96.2% (**Figure 2**). The data of HSV-2 were almost identical to those of HSV-1.

Sensitivity: Eight concentrations of PUC18-HSV type standards ranged from 5×10^1 copies/ml to 5×10^8 copies/ml and each of them was conducted by real-time PCR. Amplification curve was stably obtained when the concentration of standard samples ranged from 5×10^2 copies/ml to 5×10^8 copies/ml. However, when the concentration of standard samples was below 5×10^1 copies/ml, the real-time PCR results

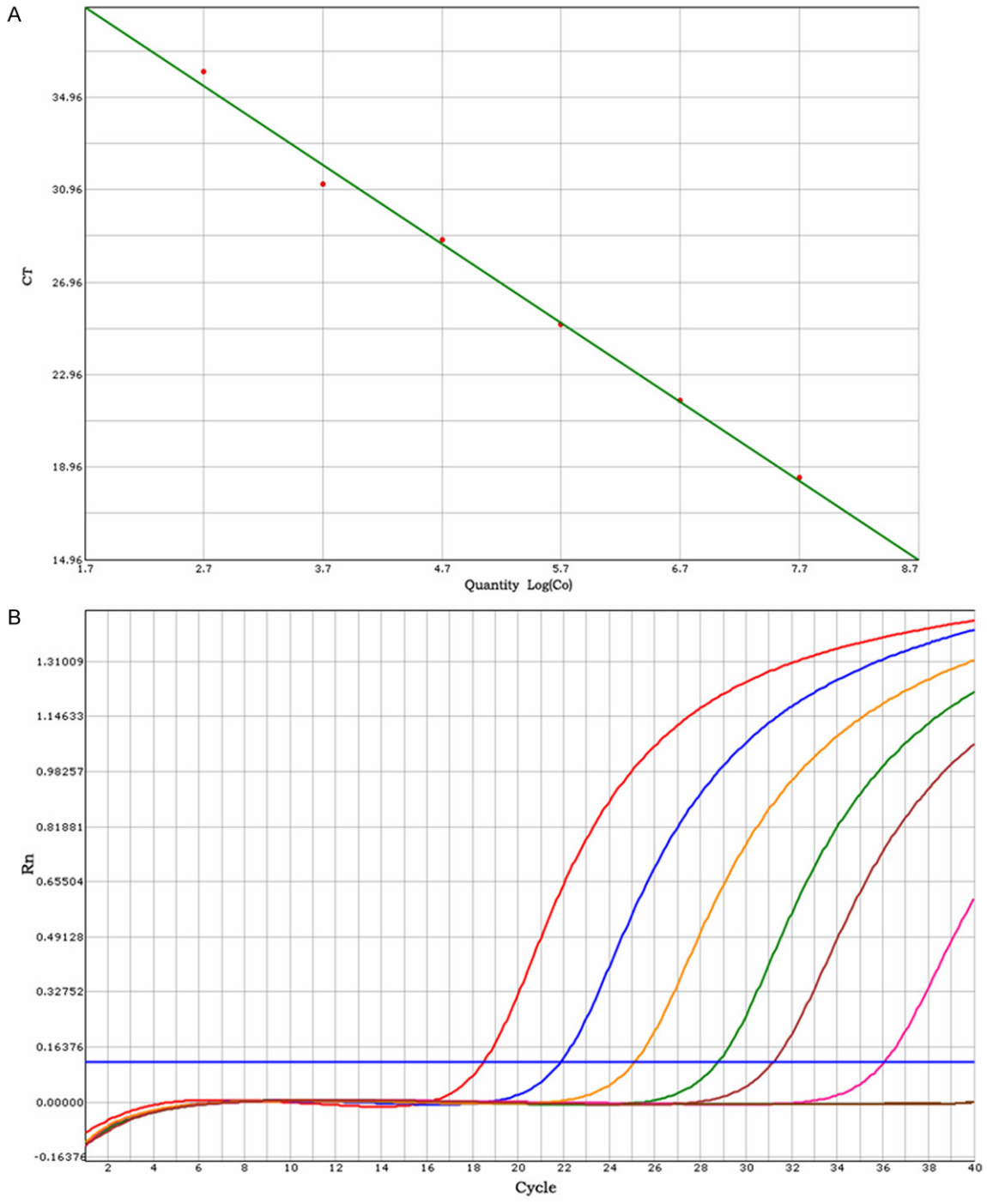

Figure 2. A. Standard curve used to calculate the quantity of HSV-1 DNA in unknown Samples. The linear regression coefficient was 0.997 and efficiency of PCR was 96.2%. B. Fluorescent curves of the standard dilution series. From the left to the right 5×10^7, 5×10^6, 5×10^5, 5×10^4, 5×10^3, 5×10^2 and the negative control is presented by the horizontal straight line.

were unstable. Thus ultimately the sensitivity of the method was 5×10^2 copies/ml.

Reproducibility: Reproducibility of intra-assay and inter-assay were conducted to the international control strains. Standard deviation (SD) and coefficients of variation (CV) were calculated on basis of cycle threshold values (Ct). The mean intra-SD was 5.41, CV was 2.29%; the inter-SD was 5.46, CV was 4.76%. The result indicated that the real-time PCR had good reproducibility.

Detection and typing of HSV in clinical specimens

186 swab specimens were tested for HSV by real-time PCR, and the positive rate was 23.7% (44/186). According to standard samples, 8 (18.2%) were positive for HSV-1 with a viral load of 8.5546×10^6 copies/ml and 36 (81.2%) were positive for HSV-2 with a viral load of 1.9861×10^6 copies/ml. The difference was significant ($P<0.05$).

To make a comparison, 186 swab specimens were tested for HSV by both viral culture and real-time PCR. The positive rate and typing of all specimens by the real-time PCR corresponded 100% with that of viral culture.

Discussion

Diagnostic methods, ranging from traditional culture and enzyme immunoassay (EIA) detection methods, to molecular techniques, have been described for the diagnosis and typing of HSV [15, 16]. Most of these assays, including the gold standard of viral isolation by culture, are slow and prone to contamination. The assay turn-around time for culture is 4 days as compared to 4 hours for EIA and 2-4 hours for real-time PCR [17]. Viral isolation diagnosis is useful if HSV is responsible for symptomatic infection in the form of vesicles or ulcers, when live virus can usually be isolated. Success of detection further depends on the secretion of virus during sampling. Its sensitivity relies on the way samples are collected, transported and stored. Viral isolation can only be done in laboratories with expertise and facilities, but in developing countries this facility may not be available [12-18].

DNA amplification using PCR technique is reported to be more sensitive than viral culture, and a number of studies have used fluorescent based real-time PCR technique with primers targeting sequences from HSV glycoprotein, thymidine kinase or DNA polymerase genes [18, 19]. In our study, by employing the real-time PCR technique, the primers and probes respectively targeted at HSV-1 DNA polymerase gene and HSV-2 glycoprotein D gene fraction were designed and applied to amplify DNA from HSV-1 or HSV-2. Evaluation on the real-time PCR method showed that specific slides were amplified from HSV-1 and HSV-2 control strains and the typing was correct. Standard curve had a good correlation when the concentration of standard samples ranged from 5×10^2 copies/ml to 5×10^7 copies/ml. Sensitivity of 5×10^2 copies/ml indicated that this method had the highest sensitivity and a good reproducibility (the mean intra-CV 2.29%; the mean inter-CV 4.76%). Our assays were able to estimate HSV load and distinguish between specific HSV-1 and -2 in swab specimens. Under the conditions used, sufficient specific amplicons were generated for accurate typing in a single step.

186 swab specimens were tested for HSV by the real-time PCR, and the positive rate was 23.7% (44/186). Among the 44 positive specimens, 8 (18.2%) were positive for HSV-1 and 36 (81.2%) were positive for HSV-2. The result showed that the incidence of HSV-1 in genitals was increasing, but HSV-2 was more commonly detected than HSV-1 (81% opposed to 18%). This is concordant with recent work that found most subjects were secreting HSV-2 [16-20]. Our results differed from those of previous studies that typically reported viral isolation sensitivities of only 58 to 79% compared to the sensitivities of a variety of laboratory-developed real-time HSV PCR assays for dermal and genital samples [21, 22]. The unusually high culture-positive rate of 100% in our study can be attributed to abundant-quality samples with high viral loads and to laboratories with expertise and facilities.

The data in this study showed that the HSV-2 viral load (8.5546×10^6 copies/ml) was higher than the HSV-1 viral load (1.9861×10^6 copies/ml) in swab specimens. The statistically significant effects suggested that subjects with HSV-2 tended to secrete more virus than those with HSV-1. Trend towards higher median HSV-2 DNA titer with the presence of lesions indicates that HSV-2 viral load plays a role in the severity of GH clinical expression [22, 23]. A study found that first episodes of GH were associated with significantly higher viral loads compared to recurrent or atypical cases [10]. Further studies with a larger sample size are required to investigate whether there is any relation between viral load and the infectivity, recurrence and prognosis of patients with GH. However, available evidence suggests that HSV viral titer in genital secretions can be a useful method for disease monitoring purposes.

In conclusion, the real-time PCR is a specific, sensitive and rapid method for the detection and typing of HSV, which can be used in clinical diagnosis and epidemiological investigation of GH.

Disclosure of conflict of interest

None.

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