Detection and Identification of Human Herpesvirus type 6 (HHV-6) Infection by Polymerase Chain Reactions and Restriction Endonuclease Analysis

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Abstract

Human herpesvirus 6 (HHV-6) is a widespread causative agent of exanthum subitum or roseola infantum. The species of HHV-6 is divided into two variants, HHV-6A and HHV 6-B, which might be detected and identified by PCR and RE methods. The three samples of eye swabs from children with suspected HHV-6 neurological illness were extracted by guanidium isothiocyanate method before running in PCR followed by agarose electrophoresis as a detection stage. The positive results were then identified by RE analysis to determine the variant of the viruses. One of the three samples (sample 2) shows positive result in direct electrophoresis and it was identified as HHV-6B which produced two fragments of 153bp and 70bp in the RE analysis. PCR and RE analysis are useful methods in detection as well as identification of HHV-6.

*Keywords:*HHV-6, PCR, Restriction endonuclease analysis

INTRODUCTION

Human herpesvirus 6 (HHV-6) is a widespread causative agent of disease in the first 2 years of life, namely exanthum subitum or roseola infantum, characterized by high fever for a few days and appereance a rash coinciding with subsidence of the fever (Yamanishi *et al.*, 1988). HHV- 6 infects almost 90% of people aged from under 10 to 59 years without showing substantial difference in the positive rate for all age group (Okuno *et al.*, 1989). The virus was initially isolated in 1986 from peripheral blood lymphocytes (PBL) of patients with lymphoproliverative disorders and immunes abnormalities (Salahuddin *et al.*, 1986). Electron microscope revealed HHV-6 is an enveloped virus, and icosahedral symmetry with 162 capsomers containing large double stranded DNA genome. (Salahuddin *et al.*, 1986, Joseph *et al*, 1986).

HHV-6 belongs to Roseolovirus genus of the β-herpesvirus subfamily and the species of HHV-6 is divided into two variants: HHV-6 variant A and B (HHV-6A and HHV 6-B) (Ablashi *et al.*, 1991). This division is also supported by other studies using monoclonal antibodies (Chandran *et al.*, 1992), PCR amplification, and DNA hybridization (Aubin *et al.*, 1990). The two variants are closely related but show consistent differences in biological, immunological, epidemiological, and molecular properties (Mori & Yamanishi, 2007).

In order to detect HHV-6 infection, there are some methods that might be approached by serological methods as well as molecular methods. Serological diagnosis is conducted by indirect immunoflourescence for the presence of antibodies to HHV-6 (Irving & Anthony, 1990). On the other hand, molecular methods for detection of HHV-6 can be conducted by PCR amplified by agarose gel electrophoresis and dot blot hybridization with a cloned DNA probe (Kondo *et al.*, 1990).

The aim of the paper is to give a brief description of detection of HHV-6 infection and identification of its variant in the communal and clinical settings by means of PCR and restriction endonuclease typing respectively.

MATERIALS AND METHODS

DNA extraction

The extraction was conducted by guanidium isothiocyanate method. Three samples used in this experiment were eye swabs in virus transport medium from children with suspected HHV-6 neurological illness. In this stage, both infected cells and distilled water would be extracted and purified as positive and negative control. For each sample to be extracted 50µl was added into a sterile eppendorf tube contaning 200 µl extraction buffer (4M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarcosyl, 1mM dithiothreitol, and 62 µg/cm^3 glycogen) before it was incubated at room temperature. After 10 minute incubation, 25µl 3M sodium acetate was

added to each tube and mixed well. To each tube, 275µl ice-cold iso-propanol was added and mixed by inversion before it was incubated at room temperature for 5 minutes. The sample was then centrifuged at 12,000g for 10 minutes and the pellet was collected carefully. Having been added with 550µl ice-cold 70% ethanol, the sample was centrifuged at 12,000g for 10 minutes. Afer centrifugation, the pellet was collected carefully and allowed to air dry for 5 minutes before it was dissolved in 50µl sterile distilled water.

PCR and agarose gel electrophoresis

To prepare PCR reaction mixture, 50µl 10x PCR buffer (1cm^3 1M tris-HCl pH 8.3; 5 cm^3 1M KCl; 200 µl 1M $MgCl_2$; 1 cm^3 2% (w/v) gelatin and 2.8cm^3 sterile distilled water stored at -20°C) was added into a 1.5cm^3 eppendorf tube. Other components (10µl deoxynucleotides, 5µl primer H6-6, 5µl primer H6-7, 277.5µl sterile distilled water, and 2.5µl taq DNA polymerase GOLD (Roche) were then added sequently. The tube was then mixed well and dispensed into 45µl amount in 0.2 cm^3 tubes. After preparing PCR reaction mixture, 5µl of DNA sample was added to a vial of reaction mixture. Each tube and prepared extacted DNA of HHV-6A and HHV-6B as control were amplified in PCR machine (Perkin Elmer 9700) with appropiate cycling programme (94°C/7 minutes; 50°C/30 seconds; and 70°C/30 seconds for a cycle and 94°C/30 seconds; 50°C/30 seconds; and 70°C/30 seconds for 40 cycles).

The oligonucleotides used as primers or probes in PCR were designated H6-6 (5'-AAGCTTGCACAATGCCAAAAAACAG-3' position 103133-103109 for variant A and 10426-104242 for variant B) and H6-7 (5'-CTCGAGTATGCCGAGACCCCTAATC-3' position 102911-102935 for variant A and 104047-104131 for variant B). Amplification using primers H6-6 led to 223bp fragment, whereas primers H6-7 allowed 220bp fragment to be amplified.

Agarose gel electrophoresis was carried out by E-gel® (invitrogen) method. To a 0.5ml eppendorf tube containing 10µl sample, 1 µl loading buffer (10mM tris-HCl pH 7.5; 1mM EDTA; 0.005% bromophenol blue) was added followed by adding 10µl sterile distilled water. Onto a 2% E-gel, 20µl of each sample was loaded and 10µl DNA molecular weight (Invitrogen) was loaded as well. To all unsused wells, 20µl sterile distilled water was loaded before the gel was run for 15-30 minutes and visualised on a transluminator.

Restriction endonuclease typing

In 0.5ml eppendorf tubes, three sets of digestion mixtures were prepared. The first set contained 5µl amplicon, 2µl RE buffer A, 1µl *AvaII* (Roche), and 12µl sterile distiled water. The second set contained 5µl amplicon, 2µl RE buffer H,1µl *HinfI* (Roche), and 12µl sterile distiled water, whereas the final set contained 5µl amplicon, 2µl RE buffer A, 2µl RE buffer H, and 13µl sterile distiled water. All tubes were then incubated at 37°C overnight. After incubation, 2µl loading buffer was added to all tubes before 10µl sample was loaded onto a 10cm^3 of 8% polyacrylamide gel (6.9 cm^3 sterile distilled water, 2 cm^3 acrylamide:bis acrylamide (37.5:1), 1 cm^3 tris-borate-EDTA buffer, 9µl TEMED, and 100µl 10%(w/v) ammonium persulphate). To one well, 10µl Hyperladder II (Bioline) as DNA molecular weight marker was also loaded. After loading samples and marker, the electrophoresis was carried out at 100V for 45 minute before it was then stained with ethium bromide and visualised on a transluminator so that variant of HHV-6 product might be determined.

RESULTS

Detection of HHV-6 infection in the samples

In this experiment, both extract positive and negative control were extracted and purified as described above in order to ensure that there is no distorsion during amplification in PCR. DNA of HHV-6A and HHV-6B was also loaded to ensure the specificity of the added primer in amplification.

From three samples assayed by PCR and direct electrophoresis, sample 2 shows the positive result of HHV-6 infection due to the same fragment as extract positive control, and both extracted DNA of HHV-6A and HHV-6B accounting for 233bp (Figure 1).

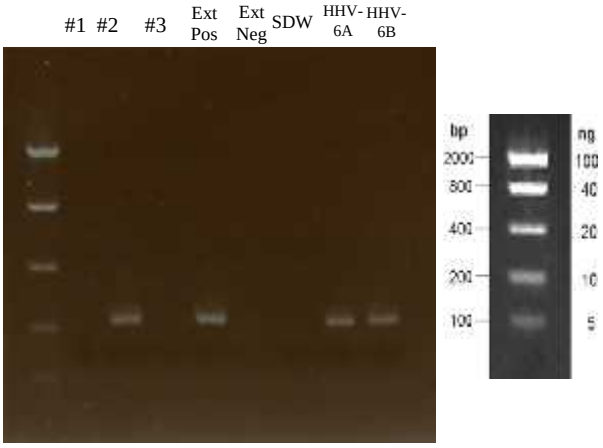

Fig 1. Separation of viral DNA by PCR and direct electrophoresis gel (left) and the DNA molecular weight markers (right). A specifically-amplified band for HHV-6 corresponds to 233 base-pairs.

Restriction endonuclease typing

In determination of variants of HHV-6 product, HHV-6 variant A is cleavaged by *AvaII* producing two fragments of 141 bp and 82 bp in the electrophoresis gel, and HHV-6 variant B

cleavaged by *HinfI* produces two fragments: 153 bp and 70 bp.

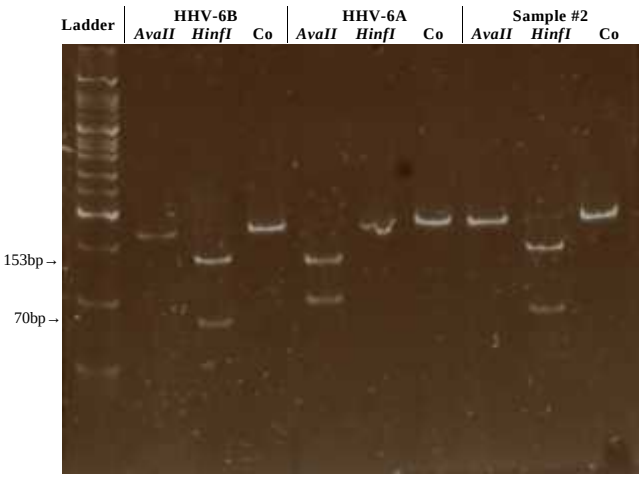

Fig 2. Identification of HHV-6 variant by RE typing. Amplicon from sample 2 has the same pattern as HHV-6B cleavaged by by *HinfI* into two fragments (153bp and 70bp).

DISCUSSION

PCR has been widely used in HHV-6 detection, particularly in clinical samples. This method might be approached to detect the HHV-6 DNA's in peripheral blood mononuclear cells (MNCs), plasma, saliva and urine of the patients (Suga *et al.*, 1995). As opposed to serological method , PCR more reliable and spesific in detection of HHV-6 infection performed only 1 to 2 days rather than 5 to 14 days in serological method (Kondo *et al.*, 1990).

In this experiment, HHV-6 infection has been detected in sample 2 by PCR and in direct electrophoresis showing positive signal in 233bp fragment. It is worth noting that there is no any fragments detected in the lane of extract negative control and distilled water indicating there is no contamination during amplification in PCR. Contamination is a major problems in amplification that can lead to false-positive results. According to Borts *et al.*, (2004) false-positive results might be caused not only by general contaminants such as reagents, laboratory disposables, equipment, or the environment, but also by sample-to-sample contamination as well as leakage between samples on agarose gels). Non spesific products owing to suboptimal assay condition may also lead to false positive results.

In order to differentiate the variant of HHV-6, namely HHV-6A and HHV-6B, restriction enzyme digestion can be performed. By using *Eco*RI, *Sst*II, *Sai*I, *Xba*I, *Hind*III, Schirmer *et al.* (1991) differentiated two different classes of HHV-6. In this experiment, *Ava*II and *Hin*fI were used to identify two variants of HHV-6. Based on the detection result, sample 2 was carried out into RE analysis and it was identified as HHV-6B due to the same restriction-fragment profile at 153bp and 70bp after digestion as HHV-6B positive control.

Although HHV-6A and HHV-6B are closely related, these two variants are consistently diferent in biological and epidemiological features. The genome of HHV-6A is 159,321 bp (Gompels *et al.*, 1995) as opposed to HHV-6B which is 161,144bp (Domiguez *et al.*, 1999). In terms of replication in in specific transformed T-lymphocyte cell lines, HHV-6A propagates in the the T-cell line HSB-2, whereas HHV-6B grows in in primary lymphocytes and in the Molt-3 or MT-4–T cells line (Mori & Yamanishi, 2007). Furhermore, there is no clear disease has yet been associated with HHV-6A, but HHV-6B is obviously the major causative agent of exanthem subitum (ES) (Yamanishi *et al.*, 1988)

PCR assay and restriction enzyme analysis are useful methods in detection as well as identification HHV-6 in terms of their sensitivity and specificity. PCR assay might be helpful, perticularly in the development of HHV-6 drugs such as acyclovir and ganciclovir, which are available for the treatment of another viruses, namely HSV, VZV, and CMV infection (Kondo *et al.*, 1990). Restriction endonuclease analysis, on the other hand, might be also helpful in determination of genetic polymorphism affecting the pathogenicity and biological properties as well as viral transmission between two strains of the same HHV-6 species (Aubin *et al.*, 1991).

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