A SENSITIVE AND SPECIFIC DETECTION OF HUMAN HERPESVIRUS 8 BY POLYMERASE CHAIN REACTION AND DOT BLOT HYBRIDIZATION

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Summary. – Polymerase chain reaction (PCR) is a powerful technique of detecting Human herpesvirus 8 (HHV-8), but has a limited sensitivity and specificity. A new assay of HHV-8 based on combination of PCR with dot blot hybridization (DBH) was developed and evaluated for its sensitivity and specificity. An HHV-8-specific primer pair, ORF26out was used for amplification of target DNA. When the PCR product was detected visually the limit of detection was 0.1 ng DNA isolated from HHV-8-infected BC-3 cells. For DBH, the DNA amplified with the primer pair ORF26in specific for HHV-8 was labeled with digoxigenin (DIG). This DIG-labeled probe was capable of detecting 1.0 ng of DNA isolated from HHV-8-infected BC-3 cells. On the other hand, PCR combined with DBH (PCR/DBH) was more sensitive than PCR or DBH alone and also very specific. The sensitivity of PCR/DBH was higher than that of PCR and DBH alone. The PCR/DBH assay can be applied efficiently to confirm the presence of HHV-8 in clinical samples and to differentiate specifically HHV-8 infection from other viral infections.

Key words: polymerase chain reaction; dot blot hybridization; Human herpesvirus 8

Introduction

HHV-8, also known as Kaposi's sarcoma-associated herpesvirus (KSHV), has been first recognized by Chang *et al*. (1994) in Kaposi's sarcoma (KS) lesions by a representative differential analysis. HHV-8 is a gammaherpesvirus (a member of the *Gammaherpesvirinae* subfamily) and the only member of human rhabdinoviruses (the *Rhadinovirus* genus, Chang and Moore, 1996; Neipel *et al*., 1997). Tumorigenic HHV-8 has a restricted host range and causes host-specific proliferative immunopathological diseases (Moore and Chang, 2001). HHV-8 is not associated with a disease in the healthy original host. However, in an immunologically ill adapted host, it is a causative agent of lymphoproliferative diseases.

Also, its natural isolates cannot be routinely propagated in cell culture (Renne *et al*., 1998). Therefore, efficient tracing of this virus relies on DNA amplification techniques. PCR represents a powerful technique of identifying viruses and studying homology between viral nucleic acids. However, PCR has a limitation due to its susceptibility to contaminants or enzymatic inhibitors (Biesiadecka and Litwinska, 1997; Broketa *et al*., 2001; Kwok and Higuchi, 1989). In order to avoid problems concerning nucleic acid amplification, efforts have been made to develop specific hybridization assays as DBH and *in situ* hybridization (McNicol and Farquharson, 1997). DBH is a simple and specific method for detection of viral DNA and has been reported as a method with higher specificity and lower sensitivity as compared to PCR assay (Duggan *et al*., 1994;

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Abbreviations: SSC = sodium citrate saline; HHV-8 = Human herpesvirus 8; PCR = polymerase chain reaction; DBH = dot blot hybridization; DIG = digoxigenin; NBT = nitroblue tetrazolium; BCIP = 5-bromocresyl-3 indolyl-phosphate; HRP = horseradish peroxidase; EBV = Epstein-Barr virus; HCMV = Human cytomegalovirus

Hwang *et al*., 1996; Xia *et al*., 1995). HHV-8 is a gammaherpesvirus, which induces frequently a latent infection, in which it is often difficult to detect HHV-8 antigens or nucleic acids because of small number of genome copies in infected tissues. Therefore more sensitive methods have been required for studying the HHV-8 pathogenesis and detection of latent infection. The use of nested PCR allows an increase in sensitivity of the virus detection. However, nested PCR assays are subjected to a high risk of contamination through DNA carry-over (Cimino *et al*., 1990; Levy *et al*., 1996; Porter-Jordan *et al*., 1990). Detection of PCR products using a non-radioactive DNA probe may provide the means for sensitive and specific detection of the target virus.

The aim of the present study was to develop a sensitive and specific assay for detection of HHV-8 for diagnostic purposes and routine screening of numerous samples obtained from the HHV-8 infection.

Materials and Methods

PCR. A HHV-8-specific PCR using the ORF26out primer pair (the forward primer 5'-AGCCGAAAGGATTCCACCAT-3' and the reverse primer 3'-TCCGTGTTGTCTACGTCCAG-5') was performed for primary amplification of target viral DNA. PCR amplification conditions were as described previously (Chang *et al*., 1994). The reaction mixture (100 µl) contained 10 mmol/l Tris-HCl pH 8.3, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.01% Triton X-100, 0.2 mmol/l dNTPs (Roche, USA), 0.2 µmol/l of each primer and 0.5 U of Taq DNA Polymerase (Roche). Thermal cycling conditions were 94°C/5 mins, followed by 35 cycles of 94°C/30 secs, 56°C/30 secs and 72°C/45 secs, and terminated at 72°C/7 mins.

Non-radioactive DNA probe specific for HHV-8 was constructed by PCR and labeled with DIG after. The HHV-8-specific PCR using the ORF26in primer pair (forward primer 5'-TATTCTGCAG CAGCTGTTGG-3' and reverse primer 3'-TCTACGTCCAGACGA TATGTGC-5') was performed as described earlier (Chang *et al*., 1994). The PCR product was purified using the Wizard PCR Preps (Promega Biotech, USA) and labeled by either random priming with DIG-dUTP (Roche) according to the manufacturer's instructions.

PCR/DBH. Detection of the PCR product was performed using the non-radioactive DNA probe. Aliquots (2 µl) of PCR mixtures after reaction were dotted on a positively charged nylon membrane (Roche). The membrane was immersed in 0.4 mol/l NaOH for 5 mins and then in a neutralizing buffer for 5 mins. After rinsing in 2 x SSC, cross-linking of the applied DNA to the membrane was carried out using a UV cross-linker (Stratagene). Hybridization solution consisted of 5 x SSC, 2% buffered blocking solution (Roche), 0.1% N-lauroylsarcosine, and 0.02% sodium dodecyl sulfate (SDS). The DIG-labeled probe, which was denatured by boiling for 10 mins and chill in ice, was added to the hybridization solution at 0.1 µg/ml concentration before hybridization. After a pre-hybridization at 50°C for 1 hr, the dot blot was hybridized at 50°C for 3 hrs and washed with 1 x SSC at 60°C for 10 mins and with a washing buffer (Roche). For detection of hybrids, the dot blot was incubated with anti-DIG conjugated with alkaline phosphatase (Roche) and then with nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolyl-phosphate (BCIP). Thereafter, the development of dark purple positive reaction was allowed to proceed for 10–30 mins in dark.

Sensitivity and specificity of PCR/DBH. To examine the sensitivity of the assay, various amounts (10, 1, 0.1, 0.01, 0.001 and 0.0001 ng) of DNA isolated from HHV-8-infected BC-3 cells (10–20 copies of the virus per cell; Arvanitakis *et al*., 1996) were subjected to PCR/DBH assay. To measure the sensitivity of primary PCR alone, 10 µl of the amplified PCR products were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide. Also, to determine the sensitivity of DBH alone, each HHV-8 DNA sample ranging from 10 to 0.0001 ng was directly applied to the membrane and then subjected to DBH. The specificity of PCR/DBH was evaluated by using samples of Epstein-Barr virus (EBV) and Human cytomegalovirus (HCMV) as template DNA, whose sequences have been reported as highly homologous to HHV-8. For this study, 10 ng of DNA of each virus was subjected to primary amplification with the ORF26out primer pair, and then DBH was carried out.

M P N 1 2 3 4 5

Fig. 1
Agarose gel (2%) electrophoresis of HHV-8 PCR products with the ORF26out primer pair

100-bp DNA ladder (lane M); positive control (P) negative control (N); 10, 1, 0.1, 0.01 and 0.001 ng of BC-3 cell DNA (lanes 1–5).

Results

When the primary amplification by PCR was performed with the primer pair ORF26out as little as 0.1 ng of DNA isolated from HHV-8-infected BC-3 cells could be detected (Fig. 1).

The strongest signal was observed with 10 ng of DNA. The sensitivity of DBH alone was low as shown in Fig. 2. The detection limit of DBH was 1 ng DNA. The strongest signal was observed with 10 ng of DNA and no signal was obtained with 0.1 ng of DNA. The increased sensitivity of PCR/DBH was apparent as the signal obtained by this technique for a given amount of DNA was always stronger than that obtained by DBH alone (Fig. 3). The specificity of

PCR/DBH was demonstrated by comparing HHV-8 with other herpesviruses with high homology in their sequences. No positive signal was seen with EBV or HCMV DNA in PCR/DBH under the conditions when HHV-8 DNA gave a strong positive signal (Fig. 4).

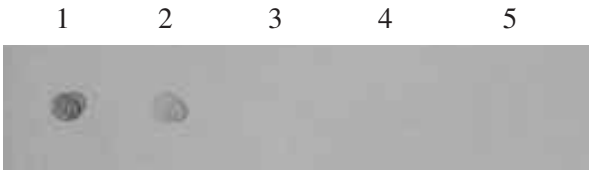

Fig. 2
DBH of HHV-8-infected BC-3 cell DNA labeled with DIG with the anti-DIG alkaline phosphate conjugate

Color reaction was obtained with BCIP and NBT. 10, 1, 0.1, 0.01, and 0.001 ng of BC-3 cell DNA (lanes 1–5).

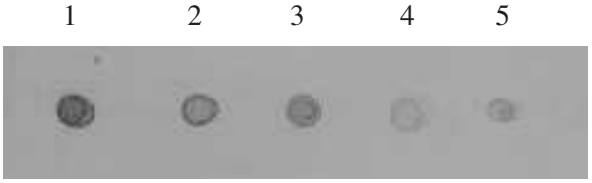

Fig. 3
PCR/DBH of HHV-8-infected BC-3 cell DNA with the anti-DIG alkaline phosphate conjugate

For the rest of legend see Fig. 2.

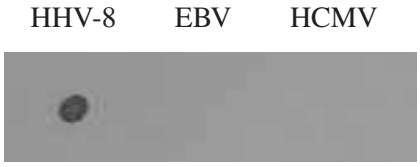

Fig. 4
Specificity of PCR/DBH

DNA from BC-3 cells infected with HHV-8 (lane HHV-8), EBV (lane EBV), and HCMV (lane HCMV). The anti-DIG alkaline phosphate conjugate used as probe.

Discussion

Diagnostics of HHV-8 has been so far dependent upon molecular biological assays because of lack of methods for antigen detection or viral isolation. Although serological assays have been developed for detection of HHV-8 antibodies, a PCR assay can be used to determine the presence of HHV-8 DNA in tissue samples and has been the only method for HHV-8 diagnostics until now (Tedeschi *et al*., 2002). However, in this study, the PCR assay could not detect HHV-8 DNA in less than 0.1 ng of DNA isolated from HHV-8-infected BC-3 cells. We need more sensitive methods for the research of its pathogenesis and latent infection. The use of a nested PCR could be an alternative tool for detection of a smaller amount of HHV-8 DNA. But, nested PCR assays have a high risk of contamination during its individual steps and may frequently result in false positive results (Chang and Moore, 1996; Neipel *et al*., 1997; Porter-Jordan *et al*., 1990). This all leads to a conclusion that it is necessary to improve the PCR sensitivity.

Therefore we attempted to develop a PCR/DBH assay for HHV-8 detection, in which the sensitivity and specificity of PCR could be increased by DBH with specific probe. We used a non-radioactively-labeled DBH probe to make this technique more attractive for diagnostic laboratories by avoiding problems related to short life radioactive compounds, their disposal, and personnel safety (Burns *et al*., 1987; Syrjanen *et al*., 1988). In the past different methods of DNA probe labeling have been developed, e.g. by radioactive isotopes (P^{32}, I^{125}) with all disadvantages mentioned above. For non-radioactive DNA probe labeling various enzymatic and immunological markers have been used (McClintok *et al*., 1991), such as horseradish peroxidase (HRP, Stome and Durrant, 1994) alkaline phosphatase (Cullen *et al*., 1997) or biotin conjugated with monoclonal or polyclonal antibodies to DNA probe. Recently, a method of non-radioactive labeling of DNA fragments with DIG-labeled dUTP randomly incorporated in DNA has been developed. DNA probe containing DIG-dUTP is detected with antibodies to DIG conjugated to alkaline phosphatase that in reaction with X-phosphate and NBT salts produces a blue-stained signal positioning the searched hybrid on the blot. The DNA probe labeling used in this study is described and the method was shown to be rapid, sensitive and specific, making it suitable for detection of amplified HHV-8 DNA products and their densitometry quantification. Complete time including the PCR and DBH procedure was 8 hrs. The PCR/DBH, developed in this study, was much more sensitive and specific as compared with the one-step PCR assay and DBH detection alone.

In conclusion, the PCR/DBH, a sensitive and reproducible method was developed for detection of HHV-8 DNA. This method may be a valid tool for a diagnostic laboratory and represents an alternative to PCR assay for screening a large number of samples for HHV-8.

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