Detection of human immunodeficiency virus, hepatitis B virus, and hepatitis C virus in donor eyes using polymerase chain reaction

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Abstract

Detection of human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) in donor eyes was performed. DNAs were extracted from the uvea, and they were amplified using the polymerase chain reaction (PCR). Amplified viral DNAs were detected with liquid hybridisation and chemiluminescent assay in which no radioactive materials were used. This method was shown to have a sensitivity limit of fewer than 10 copies of HIV, making it much more sensitive than the current techniques employed in eye banks. The method was applied to 120 donor eyes, including four from donors seropositive for HBV. The HBV gene was detected in one case in which the donor's blood had not been tested for HBV. HIV and HCV genes were not detected in any of the samples. The assay could be an effective screening test for the detection of these viruses in eye bank eyes.

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Transmission of viral diseases is one of the potential hazards associated with corneal transplantation. To date, rabies and Creutzfeld-Jakob disease have been reported to be transmitted to recipients through contaminated corneas.¹⁻⁴ For years, hepatitis B had been suspected as being transmittable and three such cases were reported (Hoft *et al*, presented as a poster at the American Academy of Ophthalmology Annual Meeting, Las Vegas, NV, 1988). Human immunodeficiency virus (HIV) has also been detected in tears, conjunctiva, and corneas from AIDS patients, although transmission via corneal transplantation has not been documented.⁵⁻⁸ Eye banks in some countries recommend examination of donor blood for HIV antibody, hepatitis B virus (HBV) antigen, and hepatitis C virus (HCV) antibody. However, the current screening method has some drawbacks. Firstly, donor blood, which is taken by cardiac, femoral, or subclavian puncture, is necessary for the detection.⁹ This procedure may be distasteful or unacceptable to family members, especially in Buddhist countries such as Japan. Secondly, false negative results are sometimes inevitable for antibody detection such as HIV. We developed highly sensitive and specific detection systems for infections with HBV, HIV, and HCV to overcome these problems. The polymerase chain reaction (PCR) method was used for this purpose. Application of the method to eye bank eyes is discussed.

Materials and methods

MATERIALS

One hundred and twenty corneas from donors who ranged from 3 to 96 years old (mean age 69.1) were examined in the study. Donor eyes were stored as whole eyeballs in a moist chamber, or in EP-II medium (Kaken Pharmacy, Chiba, Japan) until the surgery. After the preparation of a sclerocorneal button or of a whole eyeball in the case of an unused eye, the remaining portion of the eye was kept frozen at -70°C until its use for the virus detection. Three pieces of the frozen tissues weighing 1 g each, consisting mainly of uvea were excised, and they were processed as DNA source materials for detection of HIV, HBV, or HCV.

DNA PURIFICATION AND AMPLIFICATION

All the procedures were performed in a bio-hazard hood and using disposable gloves. The excised tissue was trimmed and lysed in RSB-VRC with 10% NP-40. The mixture was spun and the pellet was digested with proteinase K. The DNA was extracted with phenol chloroform and precipitated with ethanol. This extraction method yielded about 100 μg of DNA from each specimen.

PCR was performed to amplify the virus DNAs. Since the virus genes examined in this investigation were all DNAs (in HIV and HCV, virus genomes are converted to cDNA and infect target cells), the same procedure could be used for each virus, but with a different mixture of primers. Amplification primers for the HIV gag gene (SK38/SK39), the junction gene region from HBs to HBx (DNA47/DNA48), and the HCV region NS3 (A1/A2) were used. Sequences of oligonucleotide primer pairs are shown in Table 1. Each primer (100 pmol) was added per reaction. The temperature was set at 94°C during denaturation (1 minute), 55°C during annealing (2 minutes), and 72°C during polymerisation (2 minutes) on an automated thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The cycles were repeated 40 times, then the reaction was discontinued by keeping the sample at 72°C for 10 minutes. As control of PCR of genomic DNA, a 1000 base pair fragment of human β actin DNA was amplified by the DNA/PCR method using β actin primer pair (Clontech Laboratories Inc, Palo Alto, CA, USA).

DETECTION OF THE VIRUS DNA

The viral DNAs were detected from the PCR

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Table 1 Primers pairs, probes, and their sequences

Virus	Sequence of primers pair and probe
HIV	
SK38	ATAGTAGGAGCTATGTTC, 18mer
SK39	TTTCCACAGCCAGGAGTCTTGCCT, 24mer
SK19	ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC, 41mer
HBV	
DNA47	GGGGTACTTTACCGCAAGAA, 20 mer
DNA48	CCGCGTAAAGATAGGTGCGC, 20 mer
Full length	HBV DNA*
HCV	
HCVA1	CGTATGAGACACTTCCACA, 19mer
HCVA2	GCGCACACTGTGGCTTGGTA, 20 mer
HCV-A	GTCCTAGCGGCCTTGCCTACTATTCCATGG, 31mer

* HBV probe was prepared by digestion of pSHB3 with EcoRI and purified by Geneclean II (Bio 101, Inc, La Jolla, CA, USA).

products using liquid hybridisation and chemiluminescence assay as follows: the PCR samples were subjected to hybridisation with a biotinylated probe in 0.15 M NaCl buffer for 10 minutes at 95°C, then 15 minutes at 56°C. Hybridised samples were analysed by 4% agarose electrophoresis, then they were transferred to a nylon membrane using a pressure blotter (Stratagene, La Jolla, CA, USA) with 20× sodium chloride-sodium citrate. They were rinsed with 100 mM Tris-HCl at pH 7.5, then streptavidin-alkaline phosphatase was applied. The presence of the viral DNAs was confirmed by detecting the light from a chemiluminescent, Lumiphos (Sumitomo Metal Industries, Co, Tokyo, Japan). The biotinylated probe was synthesised and purified with a high performance liquid chromatography system equipped with an anion exchange column (MonoQ, Pharmacia, Uppsala, Sweden).

SELECTIVE RESTRICTION ENZYME ANALYSIS

The PCR products of HBV DNA were digested with a restriction enzyme, Bam HI, which selec-

Figure 1 Chemiluminescent detection of amplified viral DNA in HIV infected Jurkat IIIB cells (A), and PBH10R3 plasmid DNA of HIV IIIB (B). (A) Lanes 1-7: 0, 1, 2, 4, 8, 16, 32 cells, respectively. (B) Lanes 1-7: 0, 2, 4, 8, 16, 32, and 64 copies of HIV DNA, respectively.

Figure 2 Dose response detection of amplified DNAs of both HBV (A) and HCV (B). Lanes 1-6: 32, 16, 8, 4, 2, 0 copies of virus DNAs, respectively.

Bam HI :

Figure 3 Detection of the HBV DNA amplified by PCR using selective restriction enzyme analysis. Lane 1, HBV PCR positive patients; lanes 2 and 3, HBV PCR negative patients. (+)=enzyme digested DNA, and (-)=enzyme undigested.

tively cut the GGATCC sequence at 1280 of the HBV genome. The restricted sample was analysed on 1.5% agarose gel electrophoresis.

Results

CLINICAL RESULTS AND SEROLOGICAL FINDINGS

One hundred and four of 120 corneas were transplanted to recipients, while 16 were not used because of poor endothelial status, seropositivity for HBV, or simply old age of the donor. Serological examinations for HBs antigen were performed in 91 of 120 donors (75.8%), four of whom were reported to be positive. Of the 29 unexamined donors, 26 of their corneas (89.7%) were used for transplantation. Serological tests for HIV and HCV were not performed in any of the cases.

DETECTION OF VIRAL DNA BY NON-RADIOACTIVE MATERIALS

In initial studies, genomic DNA was extracted from Jurkat cells chronically infected with HIV IIIB virus and processed for the detection of HIV DNA by PCR. The PCR products were meas-

Table 2 Detection of HIV, HBV, and HCV in donor eyes

Virus studied	Number of samples in serological tests	Number of positive samples in PCR*
HIV	Not tested: 120	0/120
HBV	Positive: 4	0/4
	Negative: 87	0/87
	Not tested: 29	1/29
HCV	Not tested: 120	0/120

* The numbers indicate (number of positive samples in PCR)/(number of samples in serological test).

ured by liquid hybridisation, agarose gel electrophoresis, pressure transfer to nylon membrane, ultraviolet cross linking, and chemiluminescence assay (Fig 1). The integrated system allowed the detection of viral DNA in 3 hours.

With the rapid system, the sensitivity of the assay was examined using the diluted cell suspensions. The limitation of detection was found to be fewer than two cells, which corresponded to about eight copies of HIV DNA detected by using plasmid DNA of HIV PBH10 R3 (Fig 1). Further, we detected low numbers of the viral DNA copies (range 6–9) by HBV DNA-PCR of pSHB3 provided by Dr T Matsubara, Osaka University, Osaka (Fig 2). HCV DNA-PCR was also devised to assay for HCV provirus of eye tissue; HCV DNA was seen with as few as four to six copies of proviral DNA (Fig 2).

DETECTION OF HIV, HBV, AND HCV FROM DONOR EYES

Chemiluminescence analysis of DNA samples of donor eyes from 120 individuals revealed that one amplified DNA sample was positive for the HBV gene (data not shown). To assess the HBV DNA of the tissue, amplified HBV DNA was digested with BamHI, followed by agarose gel electrophoresis. Two digested products were generated with BamHI treatment from the HBV DNA-PCR positive eye (Fig 3, lane 1), but not from the negative eyes (Fig 3, lanes 2 and 3). The HBV positive donor had not been tested for HBV antigen before the surgery, and the corneal transplantation was performed using the tissue. The two recipients, 66 and 67 year old males, remain serologically negative for HBs antigen to date. HBV gene was not detected in four samples from seropositive donors. In contrast, DNAs of both HIV and HCV were not detected from any of the samples by the PCR assay (Table 2). However, all DNA samples of eyes yielded same PCR results of 1000 bp band with β actin primer pairs (data not shown).

Discussion

Enzyme linked immunosorbent assay (ELISA) is the commonly used screening method for infection with HIV.¹⁰ However, both false positive and false negative results can occur with ELISA. Carlson *et al* reported that six of 74 laboratory and health care personnel and 91 of 1014 blood donors were falsely positive, and four of 69 AIDS patients were falsely negative with ELISA.¹¹ Although there have been no reports to date that seroconversion developed after the transplantation of corneas from AIDS patients, seroconversion after transplantation of a contaminated kidney has been reported.^{12–14} Further, HIV was recovered in one of three corneas from patients with AIDS,⁵ suggesting that the virus is transmittable.¹⁵ Although a confirmatory test, such as western blot analysis, for ELISA positive cases is recommended, there is still the same pitfall that a negative antibody test does not necessarily mean the absence of the virus itself. Therefore, a more sensitive and specific test is necessary.

We have successfully detected the HBV gene from one donor's ocular tissue. HBV surface

antigen has reportedly been detected in cornea and tears.^{16,17} Although the use of PCR for the detection of virus gene in the cornea has been reported previously,^{18,19} this is the first report in which the HBV gene itself has been detected in ocular tissue. Detection of the virus gene by PCR appears to be a more practical and specific indicator of infection than are other techniques of detecting virus antigens. The low incidence of HBV infection determined in the present study most probably indicates that there is very little, if any, virus in the ocular tissue. One possible explanation is that the assay is not valid, although this is unlikely, since the method worked in both positive and negative controls. It seems reasonable that these viruses are less prevalent in tissues without blood perfusion, such as the cornea, unless the disease is active. In this study, none of the tested donors demonstrated active signs or symptoms of hepatitis at the time of death.

Hepatitis B surface antigen has been found in corneal donors,¹⁶ and the virus is thought to be transmittable by corneal transplantation.¹⁵ Conway and Insler investigated the prevalence of HBV infection in eye bank eyes, and reported that 69 of 5187 donors were positive for HBV antigen.⁹ The risk of HBV transmission was shown to be much greater than that for HIV transmission.²⁰ HCV is a recently isolated virus that is responsible for the majority of cases previously diagnosed as non-A non-B hepatitis.²¹ The presence of HCV in tears and aqueous humour has been reported recently, suggesting the risk of transmission of the virus through corneal transplantation.²² Because both hepatitis B and C are life threatening diseases, careful screening for the viruses is important.

Corneas from donors who test positive for infection with HIV, HBV, or HCV are not usable for keratoplasty in countries that have advanced eye bank systems. History of these viral infections is carefully taken, and appropriate serological tests are performed routinely by eye bank personnel. Unfortunately, such is not the case in many countries. Infections with hepatitis and AIDS are only screened by case history, as the serological tests are often not available.

This system presents serious public health risks, since the risk of infection of both AIDS and hepatitis through corneal transplantation is increasing.²³ This investigation was conducted as a possible prelude to the establishment of a donor screening system in eye banks. Although the results of the present study are encouraging, further studies are needed before the method supplants current serological techniques. Eye banks should have an independent examination room to avoid virus contamination of samples. Moreover, if situations allow, donor blood obtained at the time of donor eye enucleation may be a better source of DNA. The use of small amounts of blood absorbed in a cellulose sponge may be feasible and permit additional assessment of viral infection.

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