

## PROGRESS IN DIAGNOSING HERPESVIRUS INFECTIONS

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### ABSTRACT

As molecular biology has developed, several new diagnostic techniques have found application in the clinical setting. The use of the polymerase chain reaction (PCR) assay to study the molecular biology of microbial organisms has led to unparalleled advances, largely due to the rapidity with which results can be obtained. The sensitivity and specificity of PCR detection of viral DNA for diagnostic purposes are remarkable. With such excellent sensitivity, PCR is destined to become a useful diagnostic tool in herpesvirus infections. However, it is well known that herpesviruses establish latency after primary infections and that they can often be reactivated under various conditions. Because of the high sensitivity of PCR, detection of virus sequences by this method does not necessarily imply a disease state. We must be careful not to overdiagnose conditions in a clinical setting based on a PCR assay.

Key Words: herpes simplex virus, cytomegalovirus, Epstein Barr virus, HHV-6, polymerase chain reaction (PCR)

### INTRODUCTION

Of all the new methods, none has had a greater impact on medical virology than PCR, because extremely small quantities of a virus genome can be detected by PCR, regardless of the presence of any infectious virus. Following the original description of PCR in the late 1980s, this method has become a standard way for molecular biologists to probe host cell function, particularly to detect genes and the sequencing of DNA. PCR also has tremendous potential for use in the clinical environment. The sensitivity and specificity of PCR detection of viral DNA and RNA (by RT-PCR) for clinical diagnosis are excellent. Besides standard qualitative PCR detection, quantitative assays including real-time PCR can have a strong impact on the diagnosis of infectious disease.

#### (1) Herpes simplex virus (HSV)

Recently, it has been shown that extremely small quantities of HSV DNA can be detected by PCR assay, regardless of the presence of an infectious virus.<sup>1-9)</sup> Because of its excellent sensitivity, this technique may become a useful diagnostic tool for herpesvirus infections. This paper presents data on use of a PCR assay to detect HSV DNA in neonatal HSV infections and herpes simplex encephalitis.

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### A. Neonatal HSV infections

Neonatal HSV infections are severe conditions with high mortality and morbidity.<sup>10)</sup> After vertical transmission of HSV, neonates develop three types of infections according to the clinical extent of the disease (Table 1). First, HSV presents as a disseminated infection that involves several organs such as the lungs, liver, adrenals and skin with or without central nervous system (CNS) involvement. Second, HSV presents as a infection or encephalitis with or without skin involvement. Third, HSV presents as a localized skin, eye, and/or mouth (SEM) infection.<sup>11)</sup> In most cases, isolation of the virus from skin lesions or the mouth is sufficient to diagnose a neonatal HSV infection.<sup>10,12)</sup> However, skin vesicles appear in only half of the patients (Fig. 1), and viral recovery from cerebrospinal fluid (CSF) is demonstrable in only 25%–40% of neonates with encephalitis,<sup>10)</sup> and only rarely from blood analysis.<sup>13)</sup>

We applied PCR to detect HSV DNA in neonatal infections.<sup>14)</sup> For the assay, oligonucleotide primers and an internal probe were chosen from the DNA polymerase gene. When HSV DNA existed in the reaction mixture, a 330 base pair (bp) sequence was amplified by the reaction. For typing of the herpesvirus, the restriction enzymes, Xho I and Bgl II were useful for HSV-1 and -2 respectively (Fig. 2). Viral DNA was detected by PCR of specimens taken from the mouth, skin, sera and/or CSF of neonatal patients with HSV infection.<sup>14)</sup> In autopsy cases with disseminated infections, brain, lung, adrenal and liver tissues were also positive for HSV DNA. In cases of a CNS infection with skin rash, the virus was isolated from skin lesions. HSV

Table 1 Clinical Form of Neonatal HSV Infections

1	Disseminated infection that involves several organs such as the lung, liver, adrenals and skin with or without CNS involvement; Prognosis: extremely high mortality (80–90% without antiviral treatment).
2	CNS infection or encephalitis with or without skin involvement; Prognosis: 2/3 patients develop severe neurological sequelae.
3	Localized skin, eye, and/or mouth (SEM) infection; Prognosis: good.

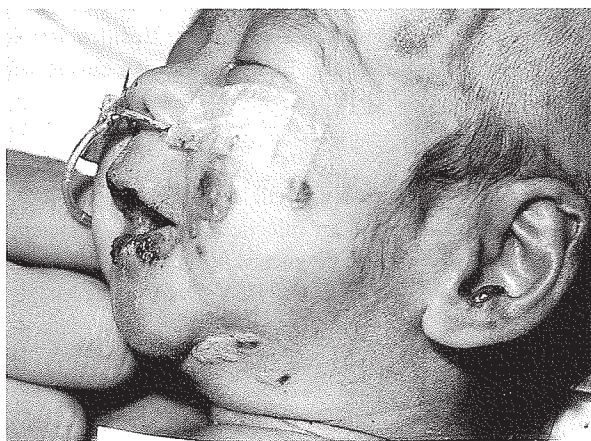


Fig. 1. A case of neonatal HSV infection with skin vesicles

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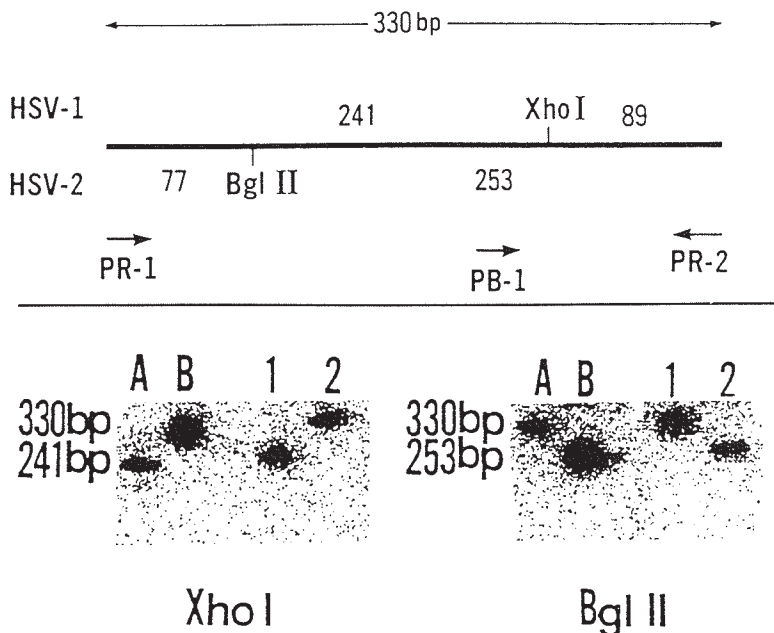


Fig. 2. HSV PCR Assay and Typing

A sequence of 330 bp was amplified by PCR. Cleavage with the restriction enzyme XhoI yielded 241 and 89 bp fragments for HSV-1, whereas BglII yielded 77 and 253 bp fragments for HSV-2. The probe (PB-1) hybridized with 241 and 253 bp fragments. Left, results from XhoI; right, results from BglII; lane A, HSV-1 standard strain KOS; lane B, HSV-2 standard strain 186; lanes 1 and 2, clinical samples; lanes 1 and 2 consisted of HSV-1 and HSV-2, respectively.

Table 2 Prolonged Presence of HSV DNA

Disseminated Form: in Serum, occasionally in CSF
CNS Form: in CSF, occasionally in serum
SEM Form: transiently, in Serum or CSF

DNA was frequently detectable by PCR in the CSF and serum samples which were negative followings virus culture. Moreover, during acute infection in disseminated cases and SEM, the HSV genome could be found occasionally in both sera and CSF samples.

In our study,<sup>14)</sup> 5 (71%) of 7 patients were positive for HSV DNA by PCR in sera and 4 (57%) of 7 in CSF. In terms of the duration of positive PCR, the prolonged presence of HSV DNA in the CSF was observed until days 7–13 of therapy. Thus, prolonged detection of HSV DNA was observed during antiviral therapy (with acyclovir or araA) in the CSF and/or sera of infants with neonatal HSV infections as shown in Table 2. In neonatal HSV infections, PCR is a useful and highly sensitive technique for diagnosis of culture-negative cases and for assessment of the prolonged presence of HSV DNA in specimens of those treated with an antiviral agent.

### B. Herpes simplex encephalitis

Herpes simplex encephalitis (HSE) is a severe disease with high mortality and morbidity<sup>15)</sup>

(Fig. 3). With the advent of effective antiviral therapy, the outcome of patients with HSV has improved,<sup>16)</sup> and early diagnosis has become more important. HSV is rarely cultured from CSF, but in cases of HSE, culturing from any other source is pointless. Intrathecal HSV antibody synthesis or the presence of virus antigens in CSF may sometimes appear negative in the early stages of infection.<sup>17-20)</sup> Isolation of HSV from brain tissue after biopsy has been considered the reference standard for the diagnosis of HSE.<sup>15)</sup> However, in several countries routine brain biopsy remains controversial.

The rapid and safe diagnosis of HSE using PCR was first reported by Rowley et al. in 1990.<sup>8)</sup> The technique showed that CSF from patients with HSE, confirmed by brain biopsy analysis or by serologic examinations, was positive for HSV DNA.<sup>8,21)</sup> Figure 4 illustrates the presence of HSV DNA in the CSF of 5 patients with HSE during its acute phase. The presence of HSV DNA in CSF continued for 3 to 18 days after the neurologic onset (mean: 10 days).<sup>22)</sup> For early diagnosis effectiveness, the detection of HSV DNA by PCR assay and HSV antibody by ELISA in CSF were compared.<sup>22)</sup> The PCR assay became positive significantly earlier than CSF-ELISA (4.4 vs 8.9 days after the onset of the disease,  $P < 0.01$ ). Figure 5 shows the time for PCR assay diagnosis of HSE. A 10 year-old boy was admitted to the Hospital 3 days after becoming unconsciousness with a fever. After the detection of HSV DNA in CSF on admission, acyclovir was administered for 2 weeks (30 mg/kg/day). PCR assay tests for HSV DNA in CSF remained positive until day 6, but turned negative on day 10, whereas an Anti-HSV antibody test on CSF was elevated on day 10.

Recently, Lakeman et al.<sup>23)</sup> reported that HSV DNA was detected by PCR assay in CSF of 53 (98%) of 54 patients with biopsy-proven HSE and was detected in all 18 CSF specimens obtained before brain biopsy from patients with proven HSE. Positive results were found in 3 (6%) of 47 patients whose brain tissue was culture-negative. The sensitivity was 95% and

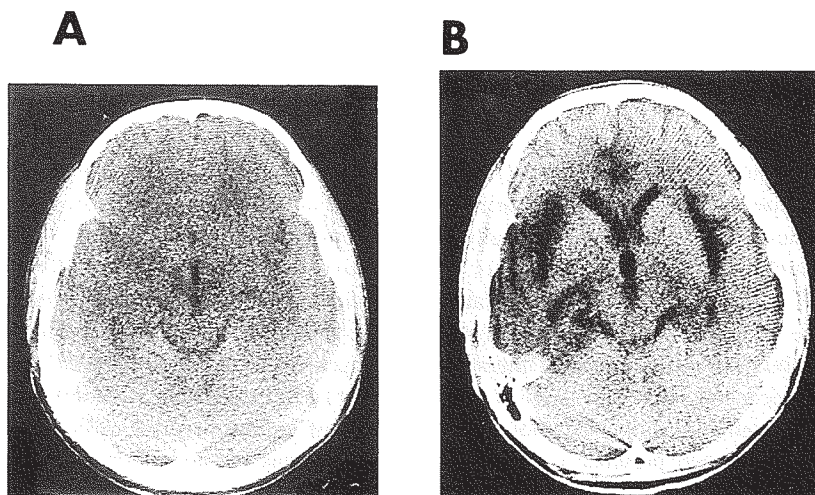


Fig. 3. Brain CT of the patient with HSE  
A: 4 days and B: 30 days after neurological onset.

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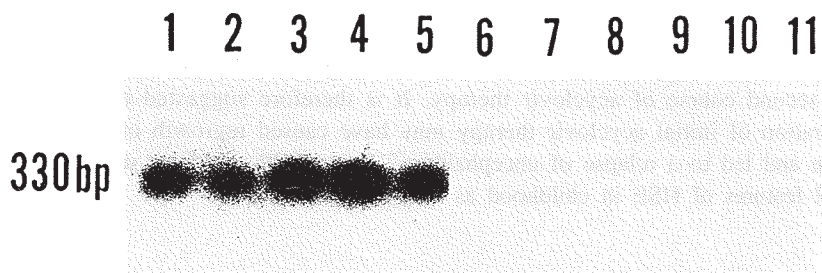


Fig. 4. HSV DNA in CSF of patient HSE  
Lanes 1-5: in five patients with HSE, HSV DNA was amplified at 330 bp before the start of antiviral therapy. Lanes 6-11: CSF of other viral encephalitis (varicella, measles, mumps, rubella).

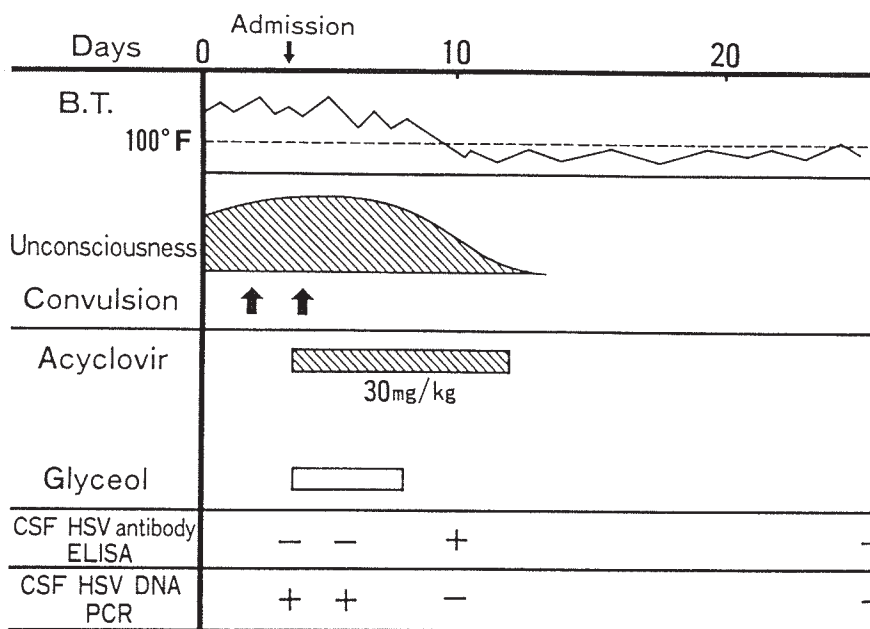


Fig. 5. Application of PCR assay for HSE diagnosis

specificity was 94%. Recently, we have also shown that specificity of the PCR assay in the diagnosis of HSE is approximately 95% (data not shown). Taken together, we conclude that PCR detection of HSV DNA should be the new standard for HSE diagnosis.

### Relapse of HSE

In our study, PCR assay was used for serial examinations of CSF samples from 15 children with HSE, four of whom had a relapse following antiviral treatment.<sup>22)</sup> Four patients had fever, altered consciousness, focal neurologic symptoms, re-elevation of white blood cells, and increased protein concentration following antiviral therapy. Serial examination by PCR assay showed that HSV DNA reappeared temporarily in 2 of the 4 recurrent cases (Fig. 6). By comparison, there was no such reappearance of virus DNA in any of the CSF for nonrecurrent patients. Interestingly, the duration of the initial treatment with acyclovir in the recurrent group was significantly shorter than that in the nonrecurrent patients. Recurrent cases also responded well to a second course of acyclovir therapy. It is therefore suggested that a comparatively shorter duration of initial acyclovir therapy may have caused regrowth of residual viruses or reactivation and led to a relapse of encephalitis.<sup>22)</sup> Using PCR, we have recently demonstrated the clinical features of HSE in childhood as shown in Table 3.<sup>24)</sup>

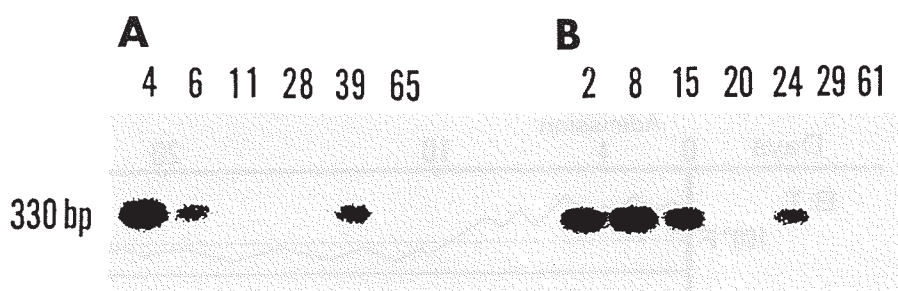


Fig. 6. Serial detection of HSV DNA in CSF of recurrent patients (ref. 22). CSF was examined by the polymerase chain reaction assay, and amplified products were shown by Southern blot hybridization. The number at the top indicates the day after onset. bp = base pairs. A: CSF was positive on days 4, 6, and 39. B: CSF was positive on days 2, 8, 15, and 24.

Table 3 Clinical characteristics of patients with PCR-proven-encephalitis

Characteristic	HSE (%)	Non-HSE (%)
Fever	100 (24/24) <sup>a</sup>	85 (32/38)
Convulsion	92 (22/24)	74 (28/38)
Initial neurologic symptoms		
Convulsion	45 (9/20)	40 (15/37)
Altered consciousness	35 (7/20)	57 (21/37)
Dysarthria	15 (3/20)	3 (1/37)
Glasgow Coma Scale on admission		
≥ 11	9 (2/23)	13 (4/29)
7-10	35 (8/23)	21 (6/29)
≤ 6	56 (13/23)	66 (19/29)

<sup>a</sup> Numbers in parentheses represent the number of total patients surveyed and those presenting the given symptom (ref. 24)

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Quantification of HSV DNA in CSF

The question arose of “how many viruses exist in the CSF of patients with herpes simplex encephalitis?” HSV DNA in the CSF of children with HSE was quantified using the PCR assay.<sup>25)</sup> During the acute phase, HSV DNA was detected in the CSF of 13 patients with HSE, including 5 neonates (neonatal HSV infection CNS form). The number of HSV DNA copies in the CSF were estimated using standard DNA fragments of HSV-1 and HSV-2. The amount of HSV DNA in the initial CSF ranged from approximately 100–100,000 copies/ml. A significantly greater number of HSV DNA copies was detected in neonates (neonatal herpes) than in older children (mean 3.9 vs. 2.5, log<sub>10</sub> copies/ml,  $p < 0.05$ ). As shown in Figure 7, serial DNA quantification showed that the HSV genome in CSF decreased along with acyclovir therapy. It has been reported that HSV replication in the brain decreased in patients receiving vidarabine therapy, but not in those that received a placebo.<sup>22)</sup> Therefore, the reduction in HSV DNA observed here was not a result of the natural course of the disease, but due to the antiviral treatment. Moreover, Domingues et al.<sup>26)</sup> suggested that the amount of viral DNA in CSF correlates with the severity of the disease. Thus a serial quantitative PCR assay is useful in estimating whether antiviral agents are effective in curbing the spread of HSV.

Quantitative PCR assay also revealed that neonates with HSE had significantly more HSV DNA copies in their CSF than older children with HSE.<sup>25)</sup> This finding concurs with other studies that show that CSF cultures from 25–40% of neonates with HSE yield virus,<sup>10,23)</sup> whereas HSV is rarely isolated from CSF in older children and adults with HSE.<sup>26)</sup> This phenomenon may be due to the permissiveness of immature brain cells, HSV replication and/or a defective immunoresponse, particularly in the cell-mediated immunoresponse among neonates.

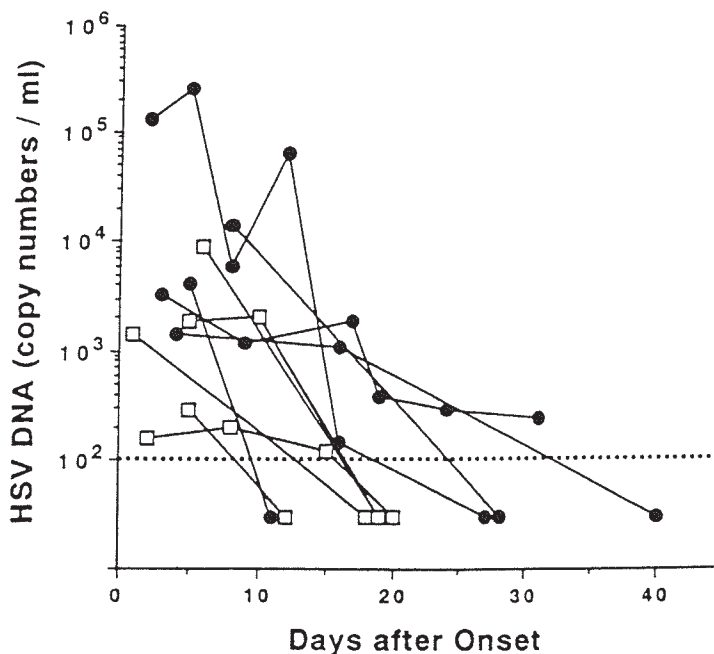


Fig. 7. Quantification of HSV DNA in cerebrospinal fluid (ref. 25)  
The number of serial HSV DNA copies in 1 ml of CSF from patients with HSE. Closed circles and open squares indicate neonates and older children, respectively. The sensitivity of the PCR assay (dotted line) was  $\sim 100$  copies/ml of CSF.

In order to clarify virus entry into CNS, the distribution of HSV DNA in neonatal HSV infections and HSE in older children was examined. The results of PCR assays in neonatal HSV infections were compared with those in older children with HSE.<sup>14)</sup> When sera were examined in disseminated cases, 8 out of 12 neonates were PCR positive, while only 1 out of 20 children ( $\geq 1$  yr old) were positive ( $p < 0.05$  as shown in Fig. 8). Hence we speculate that, first, HSV viremia may occur but disappear before the onset of neurologic manifestations in older children with HSE. Second that, in neonates, HSV may be spread principally by the hematogenous route, with subsequent replication at distant sites such as the liver, lung and/or CNS. On the other hand, HSE in older children or adults may result from intraneuronal spread of the virus, possibly without a hematogenous component.

## (2) Human cytomegalovirus (HCMV)

Anyone who is recovering from an acute infection may carry CMV in the urine, throat, and occasionally blood for months. Patients with congenital or perinatal infections and immunosuppressed patients with transplants, the human immunodeficiency virus (HIV) infection or AIDS are often chronic virus carriers for years. The isolation of HCMV from such patients requires careful clinical interpretation.

### Diagnosis of HCMV diseases

To achieve an early diagnosis, the time required has been shortened to 48 hours using cytospin and monoclonal antibody to detect viral cytopathology before it becomes visible (shell vial method). Detection of antigenemia in circulating neutrophils has been shown to be a sensitive and clinically useful method of detecting viremia.<sup>27-29)</sup> Monoclonal antibodies against a CMV matrix protein, pp65, are used in this assay. Labeled, cloned, viral nucleic acid probes have also been used to detect virus DNA or RNA in specimens by nucleic acid hybridization. Hybridization methods have been applied to clinical specimens such as buffy coats or tissue specimens.<sup>30)</sup>

Recently, PCR has been used for the diagnosis of HCMV diseases.<sup>31-33)</sup> As Spector et al.<sup>34)</sup> showed in HIV-infected patients with HCMV disease, determination of cell-free viral DNA

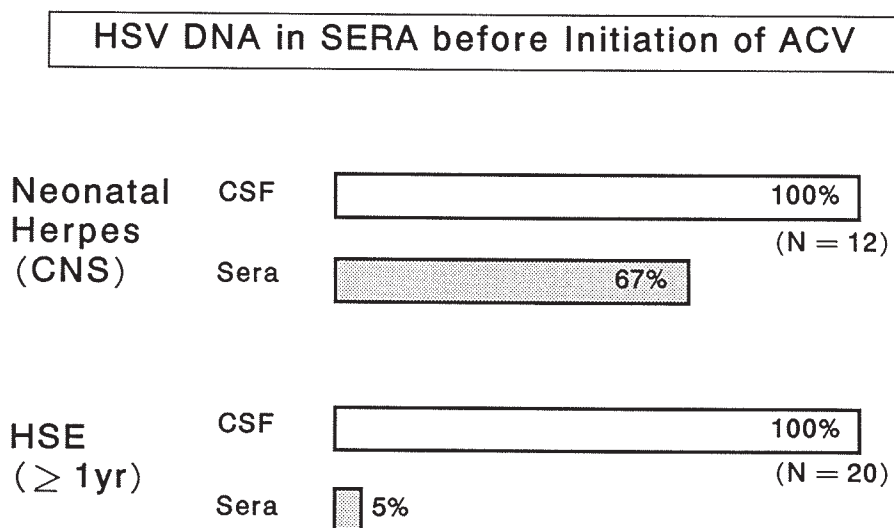


Fig. 8. Comparison of neonates and children with encephalitis in terms of HSV DNAemia



could be important in evaluating progression of the disease.

#### Quantitative PCR assay in CMV infections

In our previous study,<sup>35)</sup> quantitative PCR applied to lung tissue from bone marrow transplant (BMT) recipients found that strong PCR signals correlated with the presence of HCMV pneumonitis, determined by virus isolation and HCMV-specific histology. These results are in agreement with those of a previous report that used the conventional DNA hybridization technique to analyze human HCMV DNA in lungs from BMT recipients, and showed that a quantitative PCR assay could be useful as a diagnostic procedure for HCMV pneumonitis. A further study on quantitative PCR assay has also been reported by Rasmussen et al.<sup>31)</sup>

In their study, the CMV DNA copy number in white blood cells from both HIV-seronegative and HIV-seropositive patients was detected and quantified. Significantly, higher copy numbers of HCMV DNA were detected in HIV-seropositive patients with retinitis than in either patients with CD4 cell counts of  $<100/\text{mm}^3$  and no symptomatic HCMV disease or HIV-seropositive patients with CD4 cell counts of  $>100/\text{mm}^3$ . Prospective monitoring for increases in the HCMV DNA copy number may allow identification of HIV-seropositive patients who are at imminent risk of developing symptomatic HCMV retinitis.

#### Detection of CMV mRNA sequences

In view of the somewhat difficult interpretation of diagnostic parameters such as CMV-specific antibodies or CMV DNAemia or antigenemia, which are frequently observed in immunocompromised individuals but often associated with asymptomatic infections, the demonstration of actively replicating virus by mRNA detection could possibly allow the estimation of the relevance of the particular findings for an individual patient. With the chosen primers we detected CMV-specific mRNA sequences in PBL of actively infected patients by means of the RT-PCR technique.<sup>35)</sup> Aside from one report on the detection of MIE mRNA in 1 patient, this is the first longitudinal demonstration of CMV mRNA for both structural and nonstructural proteins by this technique during active infection in humans. Although the technique presented does not make it possible to define exactly the cell type from which mRNA was derived, the granulocyte fraction might be the main source.

These findings suggest that a certain profile of viral transcripts, which certainly has to be further differentiated, could possibly yield some kind of prognostic relevance in actively infected patients, which in turn could provide the basis for a well-calculated prophylactic treatment, perhaps with ganciclovir.

#### Studies of Organ Transplant Recipients

In the study of Meyer-König et al.,<sup>32)</sup> peripheral blood leukocytes of renal transplant recipients were investigated to compare the following markers of HCMV infection: pp65 antigen by indirect immunofluorescence, viral DNA by nested polymerase chain reaction (PCR), and immediate early (IE) and late (pp150) mRNA by nested PCR following reverse transcription. Sixty-five patients were monitored weekly for 20 weeks after transplantation. In 76 samples from 20 patients positive for HCMV DNA by PCR, HCMV mRNA was detectable amounts of IE and pp150 mRNA were positively correlated with high numbers of pp65 antigen-positive cells and confirmed the significance of the pp65 antigen as a marker for active viral replication. However, with respect to the early diagnosis of HCMV-related disease and the monitoring of antiviral therapy, the test for viral mRNA was not superior to the pp65 antigen test.

In another study by Patel et al.,<sup>36)</sup> consecutive liver transplant recipients (LTR) were studied using PCR of serum and peripheral blood mononuclear cells, reverse transcription (RT)-PCR of peripheral blood mononuclear cells, and viral blood culture for HCMV infection. These techniques were also used to predict the occurrence of HCMV infection. For diagnosis of symptomatic CMV infections, the sensitivity and specificity of the different techniques were as fol-

lows: PCR of serum, 100% and 45%; RT-PCR, 25% and 97%; PCR of peripheral blood mononuclear cells, 83% and 35%; and blood culture, 83% and 86%, respectively. PCR of serum was positive in 83% of patients with symptomatic infection before onset compared with 17% positive by blood culture. Viral blood culture remained was the best technique to diagnose symptomatic CMV infection.

Preemptive therapy is a promising approach for the management of HCMV infection in LTR. However, for this CMV therapy, it is necessary to have a laboratory marker (or patient characteristic) that identifies a subgroup of subjects at high risk for symptomatic infection, but before its occurrence, so that antiviral intervention can be maximally effective in aborting the impending disease process. From this point of view, we now apply the real-time PCR assay to monitor CMV diseases in organ and bone marrow transplant patients (Tanaka N et al. data not shown).

### (3) EB virus

Epstein-Barr virus (EBV), which is ubiquitous in humans, is a causative agent of infectious mononucleosis (IM), fatal IM, EBV-associated hemophagocytic syndrome (EBVAHS), chronic active EBV infection (CAEBV), and lymphoproliferative disorders.<sup>36-38)</sup> Primary EBV infection in infants and younger children rarely manifests as typical IM, which is a self-limiting disease.<sup>35)</sup> The last four diseases are usually severe and potentially fatal.<sup>37,40)</sup> Besides, in patients with immunodeficiency such as patients with severe combined immunodeficiency and BMT recipients, EBV could cause life-threatening diseases such as fatal IM or B-cell lymphoproliferative disorders.<sup>38-41)</sup>

Virus DNA in the peripheral blood lymphocytes of patients with IM has been detected by PCR assay.<sup>42)</sup> Virus DNA was detected in the peripheral blood lymphocytes of between 54 and 94% EBV-seropositive healthy individuals,<sup>43,44)</sup> since latently infected lymphocytes are present in about 1 in  $10^6$  lymphocytes.<sup>45)</sup> Virus DNA in the mononuclear cells (MNCs) of patients with lymphoproliferative disease was also detected and quantified as a useful marker for monitoring patients and predicting the progression of the disease.<sup>46)</sup> Recently, cell-free EBV DNA has also been detected in the serum of patients with IM.<sup>47)</sup> Here we present the quantification data of EBV DNA in plasma by PCR assay during the acute and convalescent phases of IM<sup>48)</sup> (Fig. 9). The patients included 20 patients with IM and 38 healthy children (20 EBV seropositive and 18 EBV seronegative). In patients with IM, plasma samples were positive for EBV DNA in all patients (100%) in the acute phase and in 44% of the patients in the convalescent phase, but plasma samples from the 38 healthy control children, either seropositive or seronegative, were all negative for EBV DNA (Table 1). Quantitative PCR assay revealed that plasma from patients with IM contained the highest amount of virus DNA within 7 days following the onset of the disease (mean:  $6 \times 10^4$  copies per ml). The EBV DNA concentration decreased thereafter as the patients recovered (Fig. 8). Recently, Gan et al.<sup>47)</sup> reported the detection of cell-free EBV DNA in the serum of two patients with acute IM. We confirmed the result and found that the presence of EBV DNA in plasma is a common phenomenon in patients with IM.

Quantitative analysis of cell-free EBV DNA in plasma was also applied to patients with other severe EBV-associated diseases.<sup>48)</sup> The number of virus DNA copies in plasma samples from patients with fatal IM was more than 100 times higher ( $3 \times 10^7$  copies per ml) than the average copy number in patients with nonfatal IM, suggesting that far more virus replication occurred in patients with fatal IM. EBVAHS is a nonneoplastic, generalized histiocytic proliferation with marked hemophagocytosis associated with a systemic viral infection. In the course of disease lasting several weeks to months, patients have fever, hepatosplenomegaly, pancytopenia, liver dysfunction, coagulopathy, and histiocytic hyperplasia with prominent hemophago-

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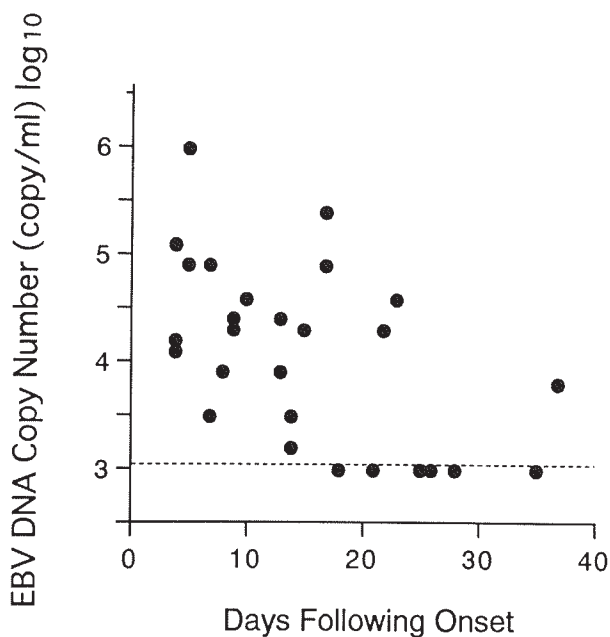


Fig. 9. Quantification of EBV DNA in IM plasma (ref. 48)

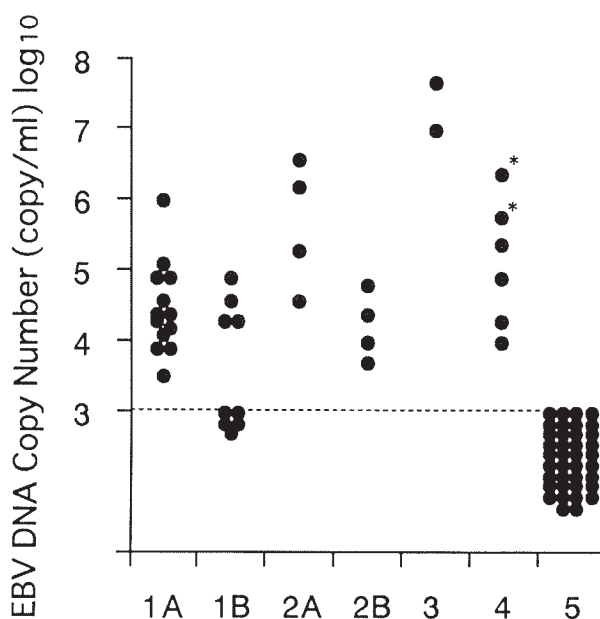


Fig. 10. Quantification of EBV DNA in the plasma of patients with various EBV-associated diseases (ref. 48) 1A, acute phase of IM (n = 13); 1B, convalescent phase of IM (n = 9); 2A, acute phase of EBVAHS (n = 4); 2B, convalescent phase of EBVAHS (n = 4); 3, fatal IM (n = 2); 4, CAEBV (n = 4); 5, healthy individuals including EBV-seropositive and EBV-seronegative children (n = 38). Two patients with CAEBV had a higher EBV DNA titer when their titer clinical state deteriorated (\*). The dashed line indicates the sensitivity of the PCR assay.

cytosis in bone marrow.<sup>39)</sup> The amount of virus DNA in patients with EBVAHS similarly decreased in the convalescent phase ( $5 \times 10^5$  copies to  $2 \times 10^4$  copies per ml). Patients with CAEBV often develop life-threatening complications over the course of months to several years.<sup>37)</sup> The clinical features are intermittent or persistent fever, lymphadenopathy, hepatosplenomegaly, and a tendency to pancytopenia and polyclonal gammopathy.<sup>37)</sup> Virus DNA copy numbers in the plasma of four patients with CAEBV were almost the same as numbers in the plasma of patients in the acute phase of IM. However, 2 patients had higher titers of EBV DNA when their clinical status deteriorated. This suggested that the viral burden was related to the clinical signs and symptoms of CAEBV.

These data suggest that the presence of cell-free EBV DNA in plasma is a common phenomenon in patients with EBV-associated diseases. The concentration of EBV DNA in plasma seems to be higher in patients with the more severe clinical categories of EBV diseases. This assay can be applied to estimate the efficacy of therapeutic agents in patients with EBV-associated diseases in the future.

#### (4) Current problems in PCR assay

For clinical application, numerous unsolved questions remain regarding use of the PCR assay. It is well known that herpesviruses establish latency after primary infection and often be reactivated under various conditions. This finding indicates that detection of virus sequences by PCR does not imply disease association caused by the viruses. PCR results often lead to misdiagnosis from this point of view. To solve this question, we propose three approaches as shown in Table 4. First, select assay materials where the virus genome is undetectable by PCR in sero-positive healthy individuals, such as CSF in HSE or serum in CMV and EBV infections. Second, use a quantitative PCR assay for any sample where, the virus might be contaminated, such as lung tissues with infiltration of infected mononuclear cells in HHV-6 related pneumonitis in immunocompromised patients. Third, samples for assay should be chosen very carefully because detection of mRNA sequences by RT-PCR also indicates active replication of the virus. Standardization of PCR assay is another important issue.<sup>49)</sup> In order to control the quality of the assay, collaborative study of each laboratory is necessary. Until then, clinical use of the PCR assay should be limited to selected laboratories.

#### (5) Recent Progress in PCR assay-qualification to quantitative assay

Real time-laser scanning coupled with a fluorogenic probe is a new technique to quantify a large number of amplified products rapidly and accurately. Using this system, it is possible to

Table 4 Important issues in clinical application of PCR assay

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1. Assay materials must be carefully selected from areas where the virus genome is undetectable by PCR, even in seropositive healthy individuals; for example, CSF in HSE or serum/plasma in HCMV and EBV infections.
  2. For samples where the virus genome may be contaminated, such as lung tissues with infiltration of infected mononuclear cells in HCMV or HHV-6 related pneumonitis in immunocompromised patients, quantitative PCR could be useful. Significantly higher amounts of viral genome in infected tissues could exclude the possibility of contamination and/or asymptotically reactivated virus.
  3. Detection of mRNA sequences by RT-PCR also indicates active replication of the virus in target cells or tissues.
  4. Standardization of laboratory PCR assay evaluation is also important.
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analyze a large number of samples within a few hours. We have shown that this system is applicable to the quantitation of EBV load in patients with symptomatic infections.<sup>50)</sup> Its accuracy and reproducibility are excellent. Furthermore, this system eliminates the precautions that must be taken with amplified products to avoid contamination because of its completely sealed condition. Our results also indicate that the real-time PCR assay is useful for diagnosing symptomatic EBV infections and for monitoring the virus load in various EBV-associated diseases such as LPD in immunocompromised patients.

## CONCLUSION

Progressing beyond simple detection of PCR products has led to the development of quantitative assays which can be correlated with outcome evaluations. These quantitative assays have now been applied to the study of HSV infections of the CNS, EBV in LPD in an immunocompromised host, and several hepatitis viruses. These techniques have also been applied to the monitoring of the therapeutic response to antiviral medications, evaluation of the predictability of PCR detection products with disease occurrence and the definition of the spectrum of illness caused by microbial agents.

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