Tracing of the molecular remnants of herpes virus infections in necrotic skin tissue

We have established an assay system to detect herpesvirus-derived transcripts in lesional crusts. Fifteen patients with herpes simplex (HS), 21 with herpes zoster (HZ), 2 with varicella, and 20 with irrelevant diseases were enrolled in the present study. Total RNA was extracted from crusts or scales, and converted to cDNA. Virus-encoded transcripts were amplified using reverse transcriptase (RT)-PCR. Housekeeping gene transcripts such as beta2-microglobulin (β2-MG) and beta-actin (β-actin) mRNA were also examined, and an efficient preservative condition of the crusts was determined. With extracted RNAs, β2-MG and β-actin mRNA were successfully amplified in all crust samples. Herpes simplex virus (HSV)-specific, lytic cycle-related transcript, UL30 mRNA was detected in all 15 HS samples, including 13 samples of HSV-1- and 2 of HSV-2-encoded UL30 mRNA, respectively. Of 23 samples, including 21 HZ and 2 varicella cases, varicella zoster virus (VZV)-specific, lytic cycle-related transcript, ORF40 mRNA was detected in 22 samples. In a control group, no UL30 and ORF40 mRNA were detected. Crust samples that had been stored without any pretreatment or preservative for 6 months at room temperature (RT) were available for the present assay. When compared with the freshly obtained materials, the amount of β2-MG mRNA was reduced to 51% in the stored samples covered with adhesive tape, to 48% in a sample left at R.T. without any treatment, and to 1.2% in the samples stocked in saline for 5 days. Herpes virus- and host-derived transcripts contained in crusts can be detected by RT-PCR amplification. Crusts or dry epidermal necrosis with inflammatory cells may provide beneficial diagnostic information.

Key words: herpes simplex virus, varicella-zoster virus, herpes simplex, herpes zoster, RNA, lytic infection

Herpes virus infection is generally diagnosed with clinical findings and the Tzanck test. However, it is sometimes difficult to discriminate herpes simplex virus (HSV) infection and varicella zoster virus (VZV) infection clinically. Although we can easily detect herpes virus in the Tzanck test, the sensitivity is low. Various microbial DNA can be detected in crusts and scales by PCR amplification [1, 2], but the positive PCR result only indicates the presence of their DNA fragments in the samples, regardless of whether the virus is pathogenic. Furthermore, it is sometimes difficult to exclude the contamination of such viruses. Especially in cases of latently infected viruses, including Epstein-Barr virus (EBV), human herpes virus (HHV)-6, and HHV-7, the detection of viral DNA does not always provide a clue to the pathogenic significance because most people carry the viruses [3-5]. The essential finding required for accurate diagnosis is to detect the involvement of such viruses in the infected sites. For this purpose, detection of herpes virus-derived transcripts, including mRNA and other virus-related RNAs, provide reliable evidence.

We have recently succeeded in detecting EBV-related transcripts in crusts from all patients with hydroa vacciniforme (HV) and EBV-associated NK/T-cell lymphoproliferative disorders [6]. To our surprise, cellular mRNAs as well as EBV-derived RNAs were preserved well enough to be detected by reverse-transcriptase (RT)-PCR in the crust, or in dry necrotic tissue. Based on these findings, we have expanded our study to establish a non-invasive, diagnostic procedure for common herpes virus infections such as herpes simplex (HS), herpes zoster (HZ), and varicella.
Materials and methods

Current diagnosis of HS, HZ and varicella was confirmed by clinical diagnosis, the Tzanck test and serological findings. Dry crusts or scales (0.5-2.0 mg) were obtained by forceps from 15 patients with HS, 21 with HZ, and 2 with varicella (figure 1). Control samples were obtained from 6 patients with HV, 3 with psoriasis, 2 with impetigo, 3 with prurigo, and 6 with insect bites. The samples were stored at room temperature (RT) until use, or if transported, they were covered with adhesive tape and mailed to our clinic without any preservative.

RNA was extracted from the samples with TRIZOL reagent (GIBCO BRL, Gaithersburg, MD), and converted to cDNA for EBV-encoded small nuclear RNA 1 (EBER1) was generated with an antisense sequence, (5′-AAAACATGC GGACCACCAGC-3′) and a random hexamer (Takara, Kyoto, Japan), in the presence of M-MLV reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA). The cDNA samples were amplified by PCR using specific primer sets for HSV-encoded UL30, VZV-encoded ORF40, and EBV-encoded EBER1 (table 1). To detect a housekeeping gene product, the beta-2-microglobulin (β2-MG) and beta-actin (β-actin) cDNA was amplified by PCR (table 1).

The PCR products were subjected to gel electrophoresis using a 2% agarose gel, and positive signals were detected by ethidium bromide staining. Direct sequencing of the PCR-amplified products was carried out to confirm the specific amplification of HSV-encoded UL30 and VZV-encoded ORF40 cDNA.

In order to find an efficient preservative condition, the amounts of β2-MG mRNA determined by quantitative RT-PCR in the same crust samples stored either in a sample left at RT without any treatment, adhesive tape, or saline condition at RT for 5 days, and were compared with those of the freshly obtained samples by real-time RT-PCR with the LightCycler system (Roche Diagnostics, Mannheim, Germany). Three specimens were analyzed and three times were calculated in each sample.

Results

With extracted RNAs, β2-MG and β-actin mRNA were successfully amplified in all crust/scale samples (figure 2). β2-MG and β-actin mRNA were detected for 6 months storage at RT (Case: HZ-4: table 2). HSV-specific, lytic cycle-related transcript, UL30 mRNA was detected in all 15 HS samples (table 2). The sensitivity and specificity of the present assay system were 100% and 100%.

![Figure 1](image1.png)

**Figure 1.** Clinical findings of HS and HZ. Each case number corresponds to Case No. in table 2.

![Figure 2](image2.png)

**Figure 2.** A representative result of our assay system. Each lane number corresponds to Case No. in table 2. Amplification gave 129bp PCR products for HSV-1 UL30 cDNA, 163bp for HSV-2 UL30 cDNA in HSV1/2 lane. M; molecular size marker.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Gen Bank accession no.</th>
<th>Sequence (5′-3′)</th>
<th>Genome coordinate</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 UL30</td>
<td>X14112</td>
<td>Sense antisense</td>
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<td>64712-64731</td>
</tr>
<tr>
<td>Ref 14</td>
<td></td>
<td></td>
<td>CCGCCCTCTCTCGGCTTCT</td>
<td>64840-64821</td>
</tr>
<tr>
<td>HSV-2 UL30</td>
<td>M16321</td>
<td>Sense antisense</td>
<td>CTGCCGAGACACCAGGGGCG</td>
<td>2143-2162</td>
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<td></td>
<td>CGACCTCCTCGGCTGCTC</td>
<td>2305-2286</td>
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<td>VZV ORF40</td>
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<td>Sense antisense</td>
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<tr>
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</tr>
<tr>
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<tr>
<td>β-actin</td>
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<td>169-189</td>
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</table>
of the 23 samples obtained from HZ and varicella, VZV-specific, lytic cycle-related transcript, ORF40 mRNA was detected in 22 samples (table 2, figure 2). The sensitivity and specificity of the present assay system were 95.7% and 97.1%, respectively, and the likelihood ratio was 33.0. Four HZ cases presented not only VZV but HSV-1 or -2. The
In a control group, UL30 and ORF40 mRNA were not confirmed in the PCR products by a direct sequencing method. In a patient with HZ (case HZ-11 (figure 2)), we could detect HSV-2 mRNA without any VZV-related transcripts. Because this patient had a lot of vesicles on the abdomen and no history of herpes simplex, we were given a diagnosis of herpes zoster clinically. But we made a final diagnosis of HSV-2 infection based on molecular evidence. Furthermore, typing of HSV was simultaneously possible by detecting the HSV type-specific transcript, UL30 mRNAs.

The HSV UL30 protein is a late protein and is essential to virus replication. The UL30 protein exhibits polymerase activity and has intrinsic 3'-5'-exonuclease activity [7]. The VZV ORF40 encodes a major capsid protein (gp42) and is a late protein in lytic infection [8]. Therefore, the presence of HSV UL30 and VZV ORF40 mRNA proves not only the infection of HSV and VZV, respectively, but also the presence of lytic cycle infection in the lesions.

One (6.7%) of the 15 crust samples obtained from HS (case HS-1: table 2) exhibited both HSV and VZV mRNA, and 4 (19.0%) of 21 samples from HZ yielded both VZV and HSV mRNA. These observations suggest the possibility that the reactivation of HSV may be induced simultaneously when VZV is led to the lytic cycle, and vice versa [9]. In addition to the detection of both HSV and VZV mRNA, IgM and IgG antibody titers against both HSV and VZV were increased in the case of HZ-21 (figure 1, table 2). These findings suggest the concomitant reactivation of both HSV and VZV, as previously reported in immuno-suppressed patients [10-12]; on the other hand, it is rarely reported in immunocompetent individuals [9, 13].

The VZV ORF40 encodes a major capsid protein (gp42), and is a late protein and is essential to virus replication. The UL30 protein exhibits polymerase activity and has intrinsic 3'-5'-exonuclease activity [7]. The HSV UL30 protein is a late protein and is essential to the cellular and herpes virus-derived RNAs are preserved in dry crusts, and that such RNAs can be detected by RT-PCR amplification with high sensitivities: 100% for HSV and 95.7% for VZV. The specificities of our assay system using a pair of specific primer sets represented 88.4% for HSV and 97.1% for VZV. In a case of HZ-11 (figure 2), we could detect HSV-2 mRNA without any VZV-related transcripts. Because this patient had a lot of vesicles on the abdomen and no history of herpes simplex, we were given a diagnosis of herpes zoster clinically. But we made a final diagnosis of HSV-2 infection based on molecular evidence. Furthermore, typing of HSV was simultaneously possible by detecting the HSV type-specific transcript, UL30 mRNAs.

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Our assay system is applicable to crust samples that have been stored under dry conditions at R.T. for many days without any pretreatment. We have furthermore confirmed mRNA existence with 2 samples saved for 6 months, as well as a sample stored for 3 months. By contrast, when the samples are stocked in saline, the amounts of detectable mRNA are markedly reduced, probably because of destruction by RNase eluted from the necrotic tissue. In a patient with HZ (case HZ-15: table 2), we could detect VZV ORF40 mRNA both in the crusty lesions obtained at the first visit (HZ-15-a: table 2) and in the scale obtained 1 month after antiviral treatment (HZ-15-b: table 2). This observation indicates that the lytic cycle transcripts of VZV might be preserved in the lesion in an intact fashion, even after antiviral treatment. As such, it appears that both pathogen-derived and cellular RNAs are well-preserved in dry, necrotic tissue, and that crusts, or dry epidermal necrosis with inflammatory cells, may provide beneficial information by means of molecular methods.

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Figure 3. The efficient preservative assay for the stock condition. β2-MG mRNA was analyzed quantitatively in comparison with the freshly-obtained samples. Black columns with bars represent means±SD of results from three repeated experiments.

Correct DNA sequence for UL30 and ORF40 was confirmed in the PCR products by a direct sequencing method. In a control group, UL30 and ORF40 mRNA were not detected in any of 20 samples. EBV-specific, latent cycle-related transcript, EBER-1 was found in all 6 HV samples, and weakly in one case of impetigo. As compared with the freshly obtained materials, the amount of β2-MG mRNA was reduced to 51% in adhesive tape or 48% in a sample left at RT without any treatment for 5 days, and to 1.2% in the same samples stored in saline (figure 3).

Discussion

Although the Tzanck test and skin biopsies are applicable for freshly isolated, living tissue specimens, it is sometimes difficult to obtain a proper tissue material for diagnostic use because herpetic vesicles soon become crust. One of the advantages of our assay system is its availability for old lesions inadequate for routine cytological examinations, even after treatment samples. This examination waits for a few days till a result is given after obtaining a specimen. For this reason the use of an antiviral therapy is recommended from a point early in time when a herpes-related disease was suspected. Detection of pathogen-derived DNA by PCR has already been applicable for the diagnosis of various microbial infections, although the PCR assay can detect viral DNA fragments and contaminated DNAs. Our assay system, however, is different from the previous method in detecting nuclear RNAs or mRNAs encoded by the pathogenic viruses by RT-PCR. This procedure, therefore, can detect the intracellular activities of the virus, which ensures the pathogenic involvement of latently or lytic-infected viruses and various host responses. In particular, most adult populations carry EBV and HHV-6 in a latent condition; it is therefore essential to detect the virus-related transcripts in the lesions. Our study has demonstrated that cellular and herpes virus-derived RNAs are preserved in dry crusts, and that such RNAs can be detected by RT-PCR amplification with high sensitivities: 100% for HSV and 95.7% for VZV. The
References


