Detection of herpes simplex virus type 1 shedding in the oral cavity by polymerase chain reaction and enzyme-linked immunosorbent assay at the prodromal stage of recrudescent herpes labialis


Recrudescent herpes labialis (RHL) is a disease caused by herpes simplex virus (HSV), predominantly type 1 (HSV-1). We have monitored HSV-1 shedding in the oral cavity by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISA) using digoxigenin-labeled primers designed to amplify a 278 bp segment of the HSV-1 UL 42 region. Prodromal RHL was confirmed by thermographic imaging in 22 patients. Infectious virus was not detected using tissue culture for virus isolation (0/22). Using PCR and agarose gel electrophoresis, we could detect HSV-1 DNA in 8/22 patients. Using a biotinylated-probe internal to the predicted sequence of the PCR product, HSV-1 DNA was detected in 10/22 of the patients by ELISA. We conclude that HSV-1 DNA is shed into the oral cavity of patients presenting with sub-clinical RHL and that the PCR-ELISA technique represents a more sensitive method to monitor HSV-1 shedding than conventional tissue culturing or PCR-electrophoresis alone.

Key words: HSV-1 shedding, PCR-ELISA; recrudescent herpes labialis; thermography

D. Scott, School of Clinical Dentistry, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BP, N. Ireland

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University of Belfast. The prodrome was confirmed by thermography and a comparison was made of the ability of tissue culture, PCR-agarose gel electrophoresis and PCR-ELISA techniques to detect HSV-1 shedding during the subclinical, prodromal stage of the disease.

Material and methods

Patient population and sampling

Twenty-two healthy patients with a known RHL history were recruited into the study; they all attended the School of Clinical Dentistry within 6 h of recognizing the prodromal symptoms of burning and/or tingling, which could be confirmed by thermography. The patients were 11 women and 11 men, with an age range of 24 to 51 years (mean 39 years). Written consent was obtained and all underwent the same protocol of thermal acclimatization in an environmentally controlled room, thermographic assessment, recording of the prodromal symptoms, and clinical evaluation of disease progression at 0 and 72 h. Sampling for tissue cultures, PCR and ELISA was by oral rinse with 10 ml phosphate-buffered saline (PBS), pH 7.2, held in the mouth for 30 s. The rinses were obtained directly following thermographic imaging. A cotton swab was taken from the prodromal region, identified by thermography, and stored in viral transport medium. There were no clinical lesions present in any of the patients enrolled at 0 h.

Infrared thermographic imaging

Thermographic assessment of the prodrome was performed using an Agema 900 thermovision system (Agema Infrared Systems AB, Danderyd, Sweden), as previously reported (17, 18). Briefly, all patients were requested to refrain from smoking, strenuous physical exercise, eating and drinking prior to thermographic imaging. Facial cosmetics were removed and no treatment was provided prior to imaging. Patients were left to acclimatize in an environmentally controlled room at 20±1°C, with no radiation sources and minimized air convection, for 20 min. Two images were recorded at a focal length of 0.5 m and of 0.05 m, which were then analyzed by the system software.

Virus isolation by tissue culture

Six tissue culture cell lines: Hep2 (human epithelial), HFF9 (human foreskin fibroblast), E6 (African green monkey), RD (human rhabdomyosarcoma), FCL (bovine fibroblast) and RMK (rhesus monkey kidney) were grown in Eagles MEM, with 10% fetal calf serum in 75 cm² plastic cell culture flasks and used for virus isolation according to the methods of O’Neill et al. (19). Monolayers were trypsin-treated every 24 h and resuspended in 5 ml growth medium. The cells were counted using a Kova counting chamber and diluted to 10⁶ cells/ml. Each of the oral rinses and cotton swabs was prepared for virus isolation by the conventional method of Schmidt & Emmans (20). Ten microlitres of each specimen was inoculated into the wells of U-bottomed tissue culture microtitre plates and four log dilutions of each specimen were made. To each specimen and dilution was added 25 µl of each of the cell culture suspensions. The plates were sealed and incubated at 37°C for up to 7 days in 5% CO₂. Monolayers had formed after overnight incubation. The plates were then observed daily for cytopathic effects using an inverted Olympus microscope at x40 magnification. HSV-1 and HSV type 2 (HSV-2) specific monoclonal antibodies (Syva) were used to identify the herpes virus in any cell lines exhibiting cytopathic effects by immunofluorescence.

PCR amplification of HSV-DNA

One hundred microlitres of each oral rinse was processed by the United States Biochemical DNA/RNA isolation kit (Amersham Life Science, catalogue no.73752), for extraction of total DNA by alcohol precipitation according to the manufacturers instructions. Viral transport medium, double-distilled H₂O and saliva from individuals with no RHL history were compared to each other and to the HSV-1 specific Ul 42 region encoding the polymerase-associated protein (22). Primer HS-14 was digoxigenin-labeled at the 5'-end.

Ten microlitres of each PCR product was loaded on a 4% agarose gel in Tris-acetate-EDTA bufer, pH 7.2 (TAE) and run at 75 V, 0.8 h, stained with ethidium bromide and examined under UV light.

DNA sequencing of PCR products

DNA sequencing was carried out to confirm the identity of the PCR products and therefore validate the use of the selected primers. A representative 278 bp PCR product from a clinical specimen and from a positive control sample, obtained through the use of primers HSV-1A and HSV-1B, and a representative 278 bp PCR product from a clinical specimen and from a positive control sample generated with primers HS-13 and HS-14, were sequenced using fluorescence-labeled deoxyNTPs by standard methods using the Perkin-Elmer ABI Prism 377 DNA sequencer. After analysis, the resulting 278 bp sequences were compared to each other and to the consensus US 42 sequence (EMBL/Genbank D10879; G221763). Following translation by the on-line Swiss-Prot system, the predicted amino acid sequences of the PCR products were also compared with the consensus sequence (Swiss-Prot P10226).

PCR-ELISA (digoxigenin detection) of HSV-DNA

Following PCR amplification as described above, all products were examined for HSV-DNA by ELISA using 5'-
bavitin-labeled probes recognizing sequences internal to the PCR products. Probe HSV-1P (5'-ACC GCC GAA CTG AGC AGA CAC-3') was specific to the 92 bp product and HS-15 (5'-ATA GTG CCA CGC CCA CGT TCG A-3') was specific to the 278 bp product. The ELISAs were carried out in microtitre plates using the Boehringer-Mannheim PCR-ELISA (DIG-detection) kit, which contains streptavidin-coated wells allowing the sequestration of the biotin-label on the HSV-IP and HS-15 probes, leaving the digoxigenin-label on the primers free for enzyme-linked colourimetric reaction. The plates were read on a Dynatech MR 700 plate reader at O.D. 405 nm with a 492 nm reference filter. An O.D. reading of three times the mean of the negative controls was considered positive for the presence of HSV-DNA.

**Results**

**Thermographic and clinical observations**

All 22 patients enrolled in the study presented with prodromal RHL confirmed by electronic infrared thermography. Typical thermograms are shown in Fig. 1, where the localized elevation in temperature, reflecting the inflammatory reaction of prodromal RHL, can be seen (Fig. 1a). Figure 1b is a thermogram of the normal appearance of the oro-labial region.

Quantitative clinical and thermographic observations of disease progression, monitored over 3 days, are presented in Table 1. The highest localized elevation in temperature, representing the RHL prodrome, seen in any patient on presentation was 1.9 °C. The highest and lowest areas of clinical lesions observed at 72 h were 200 and 0 mm², respectively, although 55% of the patients developed clinical lesions of between 10 and 30 mm².

**Detection of HSV by tissue culture, PCR-agarose gel and PCR-ELISA**

The three methods employed in the study to detect HSV-1 and/or HSV-2 are compared in Table 2.

As can be seen from the table, neither HSV-1 or HSV-2 was detected in any of the 22 oral rinse specimens, or from cotton swabs of the prodromal area, by a virus isolation technique using six different cell lines. By a virus isolation technique using six different cell lines. It should be noted that no clinically observable lesions were present at this time.

Using primer set HSV-1A and HSV-1B, which has been reported to amplify a 92 bp segment of the HSV-1 and HSV-2 DNA polymerase gene (6, 21), we apparently detected HSV-DNA in all but one of the specimens tested. All 92 bp products that were tested were also positive by ELISA using the probe HSV-1P. However, several saliva samples used as negative controls also gave a positive signal, yet sequencing of three indepen-

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Range</th>
<th>Mean</th>
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<tr>
<td>Age of patient (years)</td>
<td>24 to 51</td>
<td>39.6±9.5</td>
</tr>
<tr>
<td>ΔT °C* (0 h)</td>
<td>0.5 to 1.9</td>
<td>1.18±0.44</td>
</tr>
<tr>
<td>ΔT °C* (72 h)</td>
<td>0 to 1.6</td>
<td>0.55±0.37</td>
</tr>
<tr>
<td>Area of thermographic involvement at 0 h (mm²)**</td>
<td>30 to 461</td>
<td>116±108</td>
</tr>
<tr>
<td>Area of thermographic involvement at 72 h (mm²)**</td>
<td>0 to 255</td>
<td>56±69</td>
</tr>
<tr>
<td>Area of clinical lesion at 72 h (mm²)***</td>
<td>8 to 200</td>
<td>37±50</td>
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</tbody>
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* Difference in temperature between the normal tissue and the thermally active area.

** Thermographically measurable area of lesion.

*** Data from patients whose prodrome aborted are not included in these statistics.

Table 2. Detection of HSV-1 shedding during the RHL prodrome by tissue culture, PCR and ELISA

<table>
<thead>
<tr>
<th>Technique employed*</th>
<th>Number of samples in which HSV-1 detected</th>
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<tbody>
<tr>
<td>Tissue culture</td>
<td>0/22</td>
</tr>
<tr>
<td>PCR (primers HS13 and HS14)</td>
<td>8/22</td>
</tr>
<tr>
<td>ELISA (probe HS15)</td>
<td>10/22</td>
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* Details of each technique are given in the text.

Fig. 1. (a) Facial thermogram of a subclinical developing prodromal herpes labialis lesion taken at a focal length of 0.5 m. The lesion site is right of centre of the upper lip. The localized increase in temperature is seen as a lighter colour relative to the rest of the lip. (b) Facial thermogram of the same individual taken at a focal length of 0.5 m during a non-active phase of recrudescent herpes labialis. Note the even temperature distribution on the oro-labial region. The areas at the lip junctures are naturally warmer and therefore appear lighter.
dent PCR products derived from the use of HSV-1A and HSV-1B primers was either unsuccessful or showed no significant similarity to the expected DNA sequence corresponding to the HSV-DNA polymerase gene.

When primers HS-13 and HS-14 were employed in the PCR amplification of a 278 bp segment of the UL 42 region of the HSV-1 genome, we could detect a positive signal in 8/22 (36%) of the oral rinse specimens.

Preliminary experiments using a dilution series of a known titre of an HSV-1 isolate suggested that the ELISA system allowed for a 10-fold increase in sensitivity over agarose gel-electrophoresis in detection of the 278 bp PCR products. As shown in Table 2, we could detect HSV-1 DNA by ELISA, using probe HS-15, in 10/22 (45%) of the oral rinses.

DNA sequence analysis of PCR products

The DNA sequences of the 278 bp PCR products from a HSV-1 positive control and a clinical specimen were examined for homologies. Identities of 91.1% and of 93% were observed between the HSV-1 positive control and the clinical specimen, respectively, with the UL 42 consensus sequence. Analysis of the predicted amino acid sequences obtained from each amplicon exhibited 92% and 98% identity to the consensus sequence deposited in Swiss-Prot and accessed through the University of Geneva.

Discussion

Tissue culture using 6 different cell lines was ineffective in detecting shedding of HSV-1 or HSV-2 during the sub-clinical prodrome of recrudescent herpes labialis.

We discontinued the use of primers HSV-1A and HSV-1B, previously used to detect HSV-DNA (6, 21), as positive signals were occasionally observed in the saliva negative controls but primarily because we were unable to confirm the identity of the PCR products through DNA sequencing under the conditions of our laboratory experimental protocol.

PCR amplification of a segment of the HSV-1 polymerase-associated protein-encoding gene using primers HS-13 and HS-14 represents an excellent means by which to detect the presence of HSV-1 DNA in the oral cavity, with the sensitivity of the detection system being improved by performing ELISAs on the PCR products. A 10-fold increase in sensitivity was observed using dilutions of a known titre of a HSV-1 isolate. This system represented a reliable method for monitoring viral shedding during the prodromal phase in patients with RHL. The size of the amplicon was found to coincide with the predicted product and that of the HSV-1 positive control. The identity of the PCR product could be confirmed through the use of the biotin-labeled probe HS-15 and digoxigenin-labeled primer HS-14 by ELISA. DNA sequencing of the PCR products established that both the clinical and positive control amplicons exhibited >90% identity to the consensus sequence of the appropriate nucleotide stretch of the HSV-1 UL 42 region.

Tateishi et al. (23) have reported HSV to be present in the saliva of 4.7% of the general population by PCR in a large sample group of 1000 individuals. However, during the prodromal phase of RHL we observed that 36-45% of our patient population of RHL sufferers shed HSV-1 DNA. In agreement with our results Tateishi et al. (23) detected PCR-positive, tissue culture-negative specimens but no culture-positive specimens were PCR-negative. It has also been reported that HSV-1 DNA can be detected by PCR directly from saliva collected from patients with primary herpetic gingivostomatitis without further specimen purification steps (24). This finding may be expected, given the enormous viral load present in the saliva of patients with primary herpetic gingivostomatitis.

It is noteworthy that the age range of the patients enrolled in this study falls outside the age groups that have been reported to be the most frequent shedders of HSV-1, namely individuals aged less than 10 years and greater than 60 years (23). The large standard deviations for the thermographic and clinical measurements reported in Table 1 reflect the wide variation in disease progression observed in the 22 patients at the time of enrollment in the study.

We conclude that HSV-1 is shed into the oral cavity during the prodromal phase of RHL before a lesion is clinically apparent, but while it is thermographically detectable.

Inhibitory products in saliva (25), such as lactoferrin (26), hypothyrocyanite (27) and immunoglobulins (25), may limit detection of infectious virus by tissue culture without affecting the sensitivity of PCR. This could explain the discrepancy in the results obtained between these two detection techniques.

PCR-ELISA may be employed to evaluate HSV-1 shedding in the general population, identify basal shedding levels in known sufferers of RHL, and to investigate the shedding of HSV-1 into saliva. These findings may have implications for cross-infection and clinical trials of anti-viral drugs. Conventional tissue culture should be regarded as too insensitive to detect accurately the shedding of low levels of HSV-1 into the human oral cavity in immunocompetent patients in the absence of a clinically apparent lesion.

References


