# Detection of cytomegalovirus and Epstein-Barr virus in labial salivary glands in Sjogren's syndrome and non-specific sialadenitis

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Maitland N, Flint S, Scully C, Crean S. Detection of cytomegalovirus and Epstein-Barr virus in labial salivary glands in Sjogren's syndrome and non-specific sialadenitis. J Oral Pathol Med 1995; 24: 293–8. © Munksgaard, 1995.

To investigate the role of herpes viruses in Sjogren's syndrome, minor (labial) salivary gland tissues from Sjogren's syndrome and from non-specific sialadenitis were examined for Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) DNA by the polymerase chain reaction. Almost half of all salivary glands studied contained EBV and/or HCMV. There was, however, no significant difference between the detection of EBV or HCMV in salivary glands from patients with Sjogren's syndrome or non-specific sialadenitis. The findings are consistent with the persistence of EBV and HCMV in minor salivary glands following primary infection, but do not indicate a direct role for either virus in the aetiology of Sjogren's syndrome, and do not exclude reactivation of the viruses in this disease.

Sjogren's syndrome (SS) is an autoimmune exocrinopathy of uncertain aetiology, although a role for viruses has been proposed (1). In rats, a similar syndrome can be caused by a coronavirus (2) and in transgenic mice a similar condition associated with HTLV-1 has been described (3). Members of the herpesvirus group seem the most likely candidates as aetiological agents in the human disease, though serological studies have produced equivocal results both in relation to human cytomegalovirus (HCMV) (4–6) and to Epstein-Bar virus (EBV) (7–11).

HCMV antigens have not been identified in salivary tissue from Sjogren's syndrome patients (6). SS has occasionally closely followed primary EBV infection (12–14), suggesting EBV may be one factor initiating SS. EBV-RNA may be associated with the autoantigens SS-A (Ro) and SS-B (La) found in Sjogren's syndrome (15). EBV-DNA and early antigen (EBV-EA) may be found in Sjogren's syndrome-affected salivary tissue (16–21), though others have not found EBV-EA (22). EBV-DNA appears to be found in amounts greater than in other autoimmune diseases or normal salivary glands in some studies (16–19) but not in others (20–21). The detection of EBV-DNA in SS appears dependent on the methodology with, for example, polymerase chain reaction detecting EBV-DNA in some samples negative by *in situ* hybridization (16, 22).

To test the hypothesis that HCMV or EBV may be specifically associated with Sjogren's syndrome rather than being associated, or reactivated, in inflammatory salivary gland lesions, we have examined labial salivary gland biopsy specimens from SS and from non-specific sialadenitis for evidence of these viruses by means of the polymerase chain reaction.

## Material and methods
### Patients and tissues

Ten snap-frozen labial gland biopsy specimens were obtained from patients with secondary Sjogren's syndrome. All of the patients with Sjogren's syndrome conformed to the criteria of Fox *et al.* (11). A further 10 snap-frozen biopsies from seven patients with non-specific sialadenitis and three labial glands adjacent to excised extravasation mucoceles were used as controls.

For studies on formalin fixed material, twenty-seven labial gland biopsies from patients with a firm histological diagnosis confirming Sjogren's syndrome (23 secondary and 4 primary) and 28 biopsies with a diagnosis of non-specific sialadenitis were identified from the archives.

### Virus control templates for PCR

Virus-infected cell lines and characterised, cloned viral restriction endonuclease fragments were used as positive and negative controls for the polymerase chain reaction (PCR). These comprised:

(a) An EBV-infected human lymphoblastoid cell line (Raji strain)

(b) Wild-strain HCMV-infected human fibroblasts

(c) A clone pHS4Dhet (the complete EcoR1 Dhet EBV fragment cloned in the cosmid pHC79).

DNA from each cell line was used as the PCR-positive control target for their respective primers, and as negative control target for non-matching primers (ie Raji cell DNA with EBV primers as positive control, HCMV-DNA with EBV primers as negative control and *vice versa*). No primers cross-reactivity was detected. In addition, an internal control system to detect amplification failure was devised. This comprised a primer pair compatible with optimal buffer characteristics for the EBV and HCMV primers which detected the human genomic c-*myc* oncogene (see Table 1). Also, in preliminary experiments, sections of spleen (fixed in 10% buffered formol saline) from EBV-infected and EBV-uninfected tamarins were used as targets to optimise conditions for the PCR detection of viral DNA in formalin fixed tissues.

### Preparation of tissues for PCR

Total DNA was extracted from the whole frozen salivary gland specimens, HCMV infected fibroblasts and EBV-infected lymphocytes by homogenisation, guanidinium isothiocyanate extraction and separation on a caesium trifluoroacetate gradient (23) taking great care to avoid cross contamination. The DNA was isolated, phenol extracted, precipitated, washed in graded ethanol and redissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA pH 8.0).

Archival, formalin-fixed, paraffin-embedded biopsy material was prepared for PCR analysis in two ways.

1) Single glands were cut into 10 μ sections, dewaxed in xylene, washed in ethanol, lyophilised and incubated with a digestion buffer containing: 1% SDS

and 500 μg/ml proteinase K in TEN buffer (100 mM Tris-HCl, 40 mM EDTA, 10 mM NaCl, pH 8.0) for 24–48 h at 48–55°C until solid elements had appeared to dissolve (modified from (24)). Any debris was then removed by centrifugation and the DNA in the supernatant precipitated, ethanol washed and resuspended in 1× Taq buffer (see below) before PCR analysis. Agarose gel electrophoresis of the DNA extracts showed a streak of DNA indicating that a range of fragments from very high to low molecular weight was present. Taq buffer consisted of 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl$_2$ (optimised conditions for the multiplex PCR with several primer pairs).

2) Two or three 10 μ sections were dewaxed, ethanol washed, lyophilised and boiled for 60 min in Taq buffer covered in mineral oil to prevent excessive evaporation. The PCR reaction was then performed with the tissue still in the tube.

Both methods produced adequate PCR amplification targets from al DNA types except salivary glands without further treatments.

### Removal of salivary gland-associated PCR inhibitor

DNA to be used as a PCR target was passed through a 1 ml Sephadex G50 spun column, prepared in a sterile syringe. The column was equilibrated in 100 μl of TEN buffer (10 mM Tris HCl, 1 mM EDTA, 0.1 M NaCl pH 8.0) by centrifugation at 1600 g for 3 min at room temperature. The DNA for purification was boiled for 5 min, made up to 100 μl in TEN buffer and applied to the column. After centrifugation at 1600 g for 3 min, purified DNA was collected. Following DNA elution, the column (containing the trapped inhibitor) was "overspun" at 5000 g for 5 min to capture the inhibitor.

To check for removal of the inhibitor, an internal positive control, 500 ng of the plasmid pHS4Dhet, a construct containing the complete cloned 12.4 Kb EcoR1 Dhet fragment of EBV, which could be amplified by the EBV1 primers, was added to the extracts. Wild type EBV in the tissue extracts was then detected a second (EBV2) primer set directed against the EBV BamW repeat.

### Polymerase chain reaction

Primer pairs for the polymerase chain reaction, chosen from published sequences in the Genbank database, are presented in Table 1.

The polymerase chain reaction was performed as originally described (25) using DNA polymerase from Thermus aquaticus (Taq), in a Perkin Elmer DNA Thermal Cycler. Cycling conditions were: 9 min at 92°C, followed by 35 cycles of 1 min at 55°C, 1 min at 72°C and 1 min at 92°C, with a terminal extension phase of 7 min at 65°C. Taq polymerase reaction buffer components (particularly Mg$^{++}$ concentrations), times and temperatures for each cycle and quantity of polymerase added (2.5 units/reaction) were optimised for each primer pair. Individual optimal Mg$^{++}$ are shown in Table 1, although for multiplex analysis a compromise Mg$^{++}$ of 2.5 mM (at which all primer pairs worked in combination) was used. Positive, negative and primer-only controls were run with every batch analysed.

PCR reaction products were demonstrated by electrophoresis in 12% polyacrylamide gels prepared from a 19:1 acrylamide (BDH): bis (N,N'-methylene bisacrylamide – BDH). The final gel comprised 12% acrylamide, 0.8% ammonium persulphate (BDH), in 1×TBE buffer (0.89 mM Tris-borate, 0.89 mM boric acid, 0.01 M EDTA pH 7.8).

10 μl aliquots of reaction mixture were loaded with 10×DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll 400 in water). A marker (Phi-X 174 DNA digested with Hinf 1 endonuclease Gibco-BRL) was also run with the samples. Gels were run vertically on a BRL gel tank at 20 watts for 60 min.

When electrophoresis was complete, the gels were removed from the glass plates and stained with ethidium bromide (500 ng/ml) in double-distilled water before photographic recording. Gels were visualised by placing on a UV light source. High definition recording

Table 1. Oligonucleotide sequences of PCR primers

| PCR Product | Optimal Mg$^{++}$ | Primer sequence | DNA target | Product size (bp) |
|---|---|---|---|---|
| EBV1 | 2.5 mM | 5'-GTTCGCGTTGCTAGGCCACC-3'<br>3'-GGGACCATATTTCACCAGGA-5' | EBV BamHI w repeat sequence | 110 |
| EBV2 | 3.5 mM | 5'-CTTCAGAAGAGACCTTCTCT-3'<br>3'-ACGGACAGGCATTGTTCCTT-5' | EBV LMP gene | 197 |
| CMV | 2.0 mM | 5'-ATAATCCTGACGAGGGCCTC-3'<br>3'-TTCCTGCAGACTATGTTGAG-3' | CMV major IE gene | 200 |
| c-*myc* | 3 mM | 5'-GGAGGCTATTCTGCCCATTT-3'<br>3'-TGCAAGGAGAGCCTTTCAGA-3' | Human c-*myc* | 75 |

of gels was performed with a Polaroid MP4, rack mounted Land camera (GRI Ltd) on Polaroid 665 film.

Equivocal samples or very faint bands were tested by re-amplification for the appropriate product by cutting the region from the gel with a sterile scalpel blade. The acrylamide slice was then placed in sterile TE buffer (50 μl) and incubated at 37°C overnight allowing diffusion elution of any DNA present. The identity of CPR products of the predicted size was confirmed by direct DNA sequencing (using sequenase from US Biochemical Corp.) of purified products eluted from the polyacrylamide gels as described by MAITLAND & LYNAS (26).

## Results

### Removal of PCR inhibitor from salivary gland biopsies

Initial experiments did not produce any PCR amplification of either viral or c-*myc* DNA sequences from salivary glands. However, by introducing extra steps in the sample preparation (see Materials and methods), all of the target sequences (to a sensitivity of 10–20 copies per sample) were successfully amplified from human minor salivary glands.

Fig. 1 shows the results of a reconstruction experiment designed to confirm the presence of the PCR inhibitor in the samples The inhibitor was removed as described above and added back to half of the processed sample. The half of the sample without the inhibitor successfully amplified, whereas the reconstituted sample did not. This figure also demonstrates, for the EBV primers, that both boiling and passage through the Sephadex column are necessary to completely remove the Taq polymerase inhibitor when relatively large quantities of DNA are analysed. In subsequent experiments it was found that the simpler boil-extraction protocol also allowed successful target virus amplification from small sections. However, any increase in the target tissue mass (increasing the number of sections) caused amplification failure. HCMV primers also gave similar results using a control specimen of lung and submandibular gland from a child known to have died from overwhelming HCMV infection, confirmed by histopathology, viral culture and serology.

### Analysis of snap-frozen labial gland salivary biopsies by PCR

Employing frozen sections taken at random from a proportion of the patient group, DNA from EBV and from HCMV was detected in 50% of non-Sjogren's syndrome salivary glands, and in 40% (HCMV) and 60% (EBV) of salivary glands from Sjogren's syndrome patients (Table 2a). This prompted us to examine the available archival material for the presence of the same viral DNAs.

### Analysis of formalin-fixed paraffin-embedded labial salivary gland biopsies by PCR

To increase the number of specimens analysed, PCR analysis of the more readily available archival formalin-fixed paraffin-embedded labial gland biopsy specimens was carried out after control experiments (see Materials and methods) were performed to optimise the system.

This technique was then applied to human labial salivary gland specimens from patients with secondary Sjogren's syndrome, or from non-specific sialadenitis as controls. In this case the EBV1 primers were used to amplify target EBV sequences in the tissue, and human genomic c-*myc* oncogene primers were used as internal controls. As with the frozen human tissue analysis, successful amplification was dependent on removal of the Taq polymerase inhibitor, which co-precipitated from both fresh and formalin-fixed human tissue but not from equal amounts of formalin-fixed tamarin spleen tissue. Twenty-seven SS and 28 control non-specific sialadenitis specimens from patients whose HCMV and EBV serostatus was determined from stored serum samples, were analysed for the presence of HCMV and EBV sequences.

As shown in Fig. 2A, EBV DNA detection was readily achieved. Only samples in which both c-*myc* and the EBV primers produced positive results were scored as positive. For example, in Fig. 2A lanes 4 and 6 contain no EBV product, whereas in lane 6 a barely detectable c-*myc* signal was observed. In this case the sample was reanalysed to determine whether the lack of an EBV signal was due to a low efficiency PCR or to a genuine absence of EBV DNA. Similarly, in Fig. 2B for HCMV DNA detection, lanes 14, 16 and 17 are all HCMV negative (while c-*myc* positive) whereas lanes 5 and 6 are cases for repetition or intensification of the signal by Southern blotting and hybridization with a $^{32}$P-labelled oligonucleotide probe (33). After such confirmations, Epstein-Barr virus sequences were detected in 10/16 seropositive patients with SS and 11/15 seropositive controls. Cytomegalovirus sequences were detected in 10/14 sero-
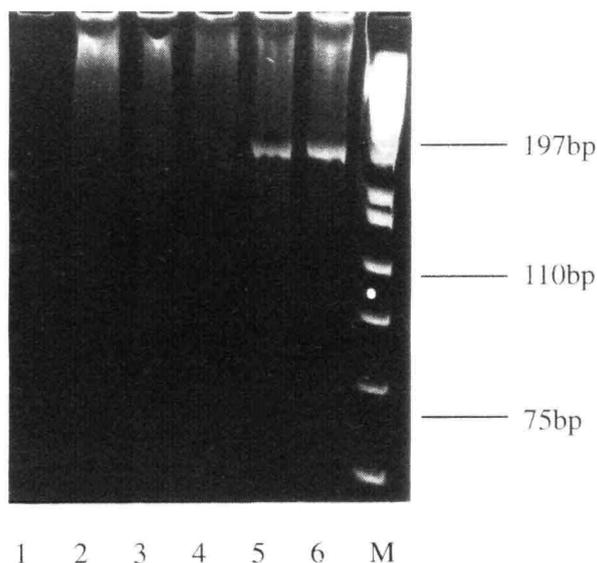


*Fig. 1. Removal of PCR inhibitor from salivary gland extracts by column chromatography.* Identical PCR reactions were set up as described in Materials and Methods, using DNA as target. All of the reactions contained the EBV1 set of primers except reaction 6, which also contained EBV2 and c-*myc* primers. After electrophoresis, the sizes of the PCR products were calculated relative to the Hinf1 digested øx174 marker (track M). The PCR reactions contained the following templates and additives: (1) No target DNA; (2) Original sample boiled and passed over Sephadex+5 μl of "column overspin" eluated; (3) Original sample digested and passed over Sephadex without boiling; (4) Original sample boiled in Taq buffer; (5) Sample boiled and passed over Sephadex; (6) As (5), but with multiple primers present (smaller products barely visible).

Table 2. Results of EBV/HCMV detection by PCR

| Diagnosis | Positive EBV by PCR | Positive HCMV by PCR |
|---|---|---|
| (a) Frozen salivary gland tissues | | |
| Sjogren's syndrome (*n*=10) | 6/10 | 4/10 |
| Non-Sjogren's syndrome (*n*=10) | 5/10 | 5/10 |
| Total | 11/20 | 9/20 |
| (b) Archival tissues | | |
| Secondary Sjogren's syndrome (*n*=23) | 16/23 | 15/23 |
| Primary Sjogren's syndrome (*n*=4) | 3/4 | 3/4 |
| Non-specific sialadenitis (*n*=28) | 15/28 | 15/28 |
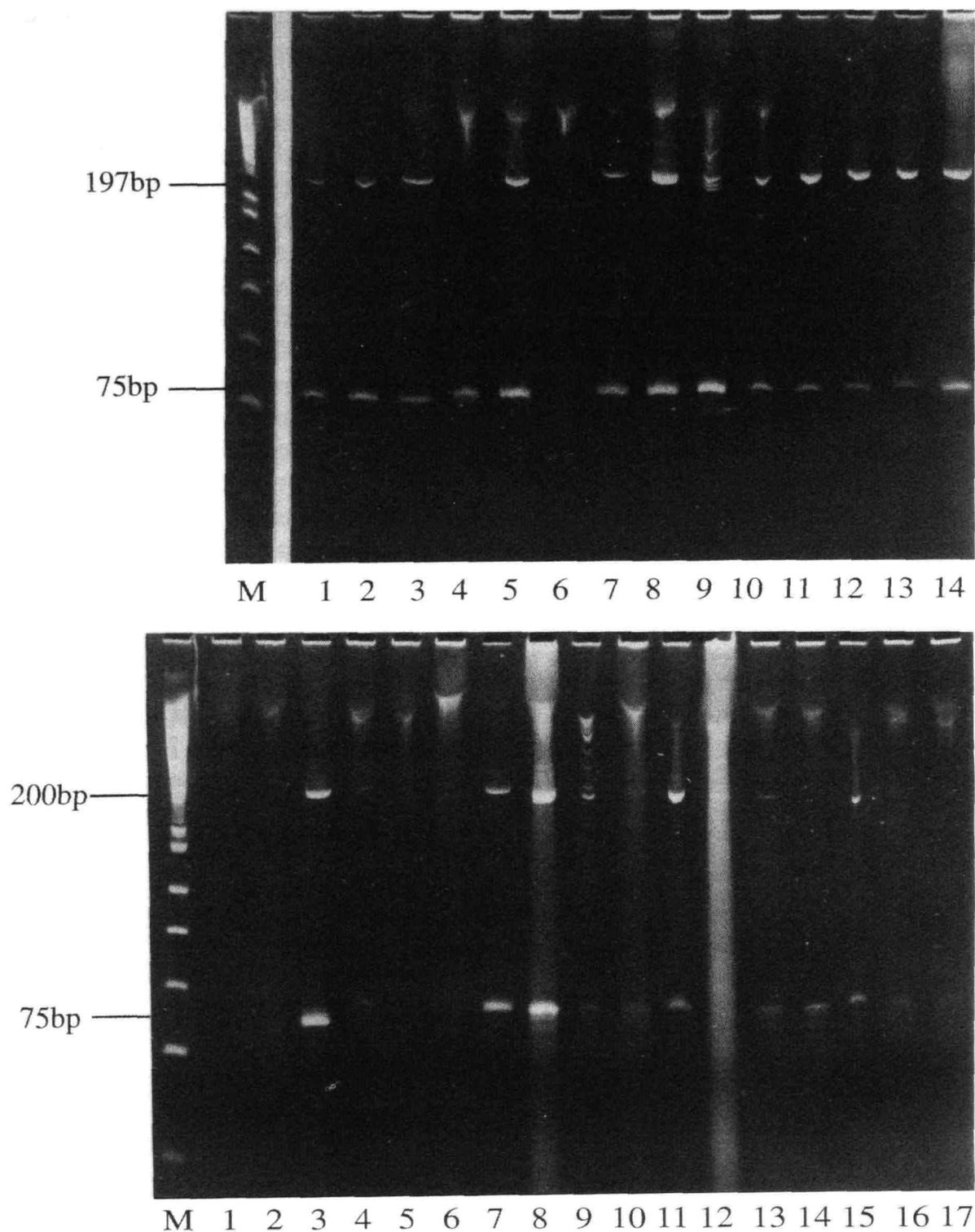
*Fig. 2. PCR analysis for the presence of EBV and HCMV in salivary tissues.* (2A) Representative analysis with multiplex EBV and c-*myc* primers. Lane M=HinfI digested øx174 DNA marker, Lanes 1–9 labial salivary gland sections, Lane 10 Tamarin spleen positive control, Lanes 11–14 labial salivary gland sections. Negative controls including 200 ng amounts of clones from other regions of the EBV, or the HCMV genome, or control human cell DNA (not shown) were consistently negative. (2B) Representative analysis with multiplex HCMV and c-*myc* primers. Lane M=øx174 marker as in 2A, Lane 1 200 ng cloned HCMV gB-1 DNA (negative control), Lane 2 200 ng pHS4DHET DNA (EBV negative control), Lanes 3–7 labial salivary gland sections, Lane 8 DNA from AD168 (HCMV) infected human fibroblasts (positive control for both HCMV and c-*myc*), Lanes 9–17 labial salivary gland sections.

breakages induced by formalin fixation leave the target sequences intact, amplification into the detection range was possible.

We have detected HCMV and EBV-specific DNA sequences in labial (minor) salivary gland tissues both from patients with Sjogren's syndrome (SS) and from non-specific sialadenitis. To optimise our chances of detecting latent viral DNA, we have deliberately overloaded the PCR and gel electrophoresis systems with DNA extracted from salivary glands, which in Fig. 2 has led to distortion of the PCR product bands. In the biopsies containing large amounts of viral DNA this distortion can be titrated out without loss of signal. Data from other viral systems (26, 28) has shown this to be a valid approach for the detection of DNA from latent as opposed to productive infections.

The cell type harbouring HCMV is unknown, but the latency site for EBV may be the epithelial elements of the salivary tissues (9, 10). The possibility also exists that we have detected EBV in infected B-cells in the lymphoid infiltrate. However, *in situ* hybridization data, using strand-specific riboprobes generated from the regions of EBV and HCMV amplified in this study, indicated that the EBV DNA in these samples was strongly associated with ductal regions and not lymphocytes (29).

The minor salivary glands are therefore a possible site of latency of these and other viruses, although the present findings neither confirm nor refute a direct association of HCMV or EBV with Sjogren's syndrome (or equally with non-specific sialadenitis). However, it may be that the clinical picture termed Sjogren's syndrome is the common result of various aetiological factors and that various different viruses might be a trigger in genetically susceptible patients, or may reactivate (30, 31). Indeed, a wide range of viruses including HCMV, EBV, hepatitis C virus and more recently various retroviruses have been implicated (32–40). A salivary gland syndrome resemling SS has been described in HIV patients (35) and those infected with HTLV-1 (36–39).

Finally, there is another sialotropic herpesvirus that should be considered: human herpesvirus 6 (HHV-6) (40). Raised serum antibody levels to HHV-6 have been found in Sjogren's syndrome (41, 42) and HHV-6 DNA has been found in salivary glands (43). HHV-6 DNA has also been detected in

positive patients with SS and 7/11 controls (Table 2b).

Chi-squared analysis of the results showed no significant difference between the presence of Epstein-Barr virus DNA either separately or in combination with human cytomegalovirus DNA in the minor salivary glands of patients with Sjogren's syndrome and controls.

### Discussion

The existence of a tissue inhibitor of PCR in the fixed salivary tissue pre-sented considerable technical difficulties at the outset of this study. It appears to be tissue specific as indicated by its absence in the other tissues studied. A similar inhibitor has also been observed in human blood (27). To achieve consistent results, it was important to optimise the balance between Taq inhibitor dilution (or removal), target sequence concentration and the target primer ratio in the reaction. Formalin fixation causes breakage of DNA and protein cross-linking, but the PCR technique requires only short unique target sequences and, so long as the random

lymphomas in Sjogren's syndrome (44, 45). However, causal relationships have yet to be established.

Thus, although several herpesviruses may latently infect salivary glands, any individual or collective role for these retroviruses or other agents singly or multiply in disease remains to be confirmed.

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