J Periodont Res 2004; 39; 81–86
Printed in the UK.

JOURNAL OF PERIODONTAL RESEARCH

Real-time PCR quantification of cytomegalovirus in aggressive periodontitis lesions using TaqMan technology

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Kubar A, Saygun I, Yapar M, Özdemir A, Slots J. Real-time PCR quantification of cytomegalovirus in aggressive periodontitis lesions using TaqMan technology. J Periodont Res 2004; 39: 81–86. © Blackwell Munksgaard, 2004

Background: Herpesviruses are implicated in the pathogenesis of human periodontitis. However, the quantity of herpesviruses in periodontal sites remains unknown.

Objective: The aim of this study was to compare levels of subgingival human cytomegalovirus (HCMV) in aggressive periodontitis patients and in periodontally healthy subjects.

Methods: A total of 16 consecutive subjects with aggressive periodontitis and 15 healthy control subjects were included in the study. Subgingival specimens were collected by a periodontal curette. TaqMan real-time polymerase chain reaction (PCR) assay was used to quantify HCMV.

Results: HCMV was detected in 68.8% of aggressive periodontitis lesions but not in any of the periodontally healthy study sites. HCMV viral load in positive subgingival specimens ranged from 5×10^2 to 7.4×10^3 copies/ml.

Conclusions: The TaqMan real-time PCR technology seems to provide a rapid and sensitive method for quantifying HCMV in periodontal pockets. The present findings confirm the frequent presence of HCMV in aggressive periodontitis lesions. Determining HCMV levels in different types of periodontitis may help elucidate the periodontopathic role of the virus and advance our understanding of the disease pathogenesis.

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Key words: aggressive periodontitis; cytomegalovirus; real-time PCR; TaqMan

Accepted for publication September 7, 2003

Aggressive types of periodontitis generally affect 0.1–2% of adolescents and young adults, although prevalences as high as 6–7% have been reported in some developing countries (1). Aggressive periodontitis has a complex pathogenic microbiota, consisting mainly of gram-negative anaerobic bacteria (2). Human cytomegalovirus (HCMV) and other herpesviruses have recently emerged as putative pathogens of the disease. Ting *et al.* (3) and Michalowicz *et al.* (4) found periodontal HCMV and other herpesviruses to be associated with aggressive localized periodontitis. Velazco *et al.* (5) detected HCMV and Epstein–Barr virus type 1 (EBV-1) in advanced periodontitis lesions of an 11-year-old patient with Papillon–Lefèvre syndrome. Nowzari *et al.* (6) identified HCMV in periodontitis lesions of an 11-year-old girl with Fanconi's anemia. Kamma *et al.* (7) studied subgingival samples from two

deteriorating and two stable periodontitis sites in each of 16 aggressive periodontitis patients who during the maintenance phase of therapy experienced progressive disease. HCMV, EBV-1 and herpes simplex virus occurred with higher frequency in periodontitis active than inactive sites, and co-infection with any of the three test herpesviruses was detected in 44% of active vs. in 3% of stable sites. Recent studies have shown that HCMV periodontal presence is associated with an increased occurrence of subgingival *Porphyromonas gingivalis* (8) and *Dialister pneumosintes* (9), two major periodontopathic bacterial species. Slots and Contreras (10) hypothesized that herpesviruses contribute to periodontal pathosis by impairing local host defenses resulting in increased virulence of resident bacterial pathogens, or by inducing the release of cytokines and chemokines from inflammatory or connective tissue cells. Recently, HCMV active infection has also been implicated in the pathogenesis of symptomatic endodontic lesions (11).

Molecular testing has become increasingly important in the diagnosis and monitoring of patients affected by viral diseases, and all herpesviral studies cited above were carried out using polymerase chain reaction (PCR)-based detection. However, because of its high sensitivity, PCR detection may show viral presence in patients with asymptomatic infections that never progress to disease. Therefore, it is important to quantify viral loads in order to differentiate clinically significant vs. latent herpesvirus burdens. Real-time quantitative PCR is highly accurate and less labor-intensive than previous quantitative PCR methods (12, 13). Also, unlike other quantitative PCR methods, real-time PCR does not require post-PCR sample handling, preventing potential PCR product carry-over contamination and resulting in much faster and higher throughput assays. The TaqMan® real-time PCR assay generates a signal by cleaving a target-specific fluorogenic oligodeoxynucleotide probe during amplification (14). Since amplification products are measured during the exponential phase of DNA amplification regardless of the initial target concentration, the TaqMan method provides exquisite sensitivity and a broad dynamic range.

The present study employed the TaqMan real-time PCR assay to compare subgingival HCMV genomic counts in aggressive periodontitis patients and in healthy control subjects. To our knowledge, this is the first investigation using a quantitative PCR method to determine HCMV levels in subgingival sites.

Materials and methods

Patient selection and clinical procedures

The present study included 16 consecutive patients with aggressive periodontitis (seven women, nine men; age 17–29 years, mean age 24.1 ± 3.3 years) and 15 healthy individuals (six women, nine men; age 21–31 years, mean age 24.1 ± 3.4 years). Patients diagnosed as having aggressive periodontitis were less than 35 years of age and exhibited probing attachment loss in the excess of 5 mm in more than 14 teeth; at least three of which were not first molars or incisors. All patients were systemically healthy and had not received periodontal treatment or antibiotics for at least 6 months prior to the clinical examination and virologic sampling. The study protocol was approved by the Ethical Committee of Gülhane Military Medical Academy, Sciences of Dentistry, Ankara, Turkey. All patients provided written informed consent after the procedures were fully explained.

The clinical examination included Plaque Index of Silness and Löe (15), Gingival Index of Löe and Silness (16), probing pocket depth and probing attachment level. All clinical measurements were performed on six sites per tooth, namely mesio-facial, mid-facial, disto-facial, mesio-lingual, mid-lingual and disto-lingual. Probings were carried out using a calibrated Williams probe.

Sample collection and preparation

Prior to sampling, supragingival plaque was gently removed with sterile cotton pellets, and the sample sites were isolated with cotton rolls and dried before removing subgingival specimens by means of a sterile curette. After gently inserting a sterile periodontal curette to the bottom of each test periodontal pocket, subgingival deposits were removed by a single stroke of the curette. In each periodontitis patient, subgingival samples collected from the three deepest periodontal pockets of the dentition (7–10 mm probing depth) were pooled into 500 µl of TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA, pH 8). In each healthy patient, subgingival samples collected from three shallow periodontal sites (2–3 mm probing depth) were pooled into 500 µl of TE buffer. After vigorous vortex mixing, 60 µl aliquots of the specimens were subjected to DNA extraction as described elsewhere (17). Extracted DNA was diluted in 30 µl of distilled water, and 10 µl of the DNA solution was used in real-time PCR examination.

Real-time PCR

The sequences of TaqMan PCR primers and probe were selected from the immediate early protein gene (locus IE) using the Primer Express Software (Applied Biosystems, Foster City, CA, USA). The sequences of the forward and reverse primers were 5′-ATCGGACTTCGYCGCGATTTGCTT 3′ (Y = C or T) and 5′-CAG ACGATCCRATGAACGTCGTTTT TTGT-3′ (R = A or G), respectively. The TaqMan probe was fluorescence labeled at the 5′ end with 6-carboxyfluorescein (FAM) as the reporter dye and at the 3′ end with 6-carboxytetramethyl-rhodamine (TAMRA) as the quencer dye (5′-FAM-AGATTTCC GCTACATTTGTCGGGACGAGG-TAMRA). TaqMan PCR was performed with a final volume of 50 µl PCR mixture, containing 25 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 400 nM (final concentration) of each of the primers, 250 nM TaqMan probe, and 10 µl of extracted subgingival sample. Primers and probe had been previously titrated to check for amplification efficiency. Amplification and detection were performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems) by using the manufacturer's standard protocols. PCR amplification, which was per-

formed in separate tubes, took place at 2 min at 50°C to eliminate carryover contamination, at 10 min at 95°C to activate the hot start *taq* polymerase (AmpliTaq Gold® DNA Polymerase, Applied Biosystems), and then at 40 cycles, with each cycle consisting of a step at 95°C for 15 s, followed by a step at 60°C for 1 min. All standards (obtained from Roboscreen, Leipzig, Germany), controls and clinical specimens were run simultaneously.

Amplification, data acquisition and all analyses were performed by using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Standard procedures for the operation of the model 7700 system were followed, including the use of all default program settings. TaqMan PCR products were detected as an increase in fluorescence from cycle to cycle. The software generates two detection responses, i.e. the fluorescence intensity by a normalized reporter value (ΔR_n) and the threshold cycle (C_t). The ΔR_n value is obtained by subtracting the value for the reporter signal R_n^+ (emission intensity of the reporter), from the value for the background signal, R^-_n [emission intensity of the reporter (no template)]. The C_t value is the cycle number at which the reporter fluorescence is greater than the fixed threshold fluorescence, a parameter defined as 10 times the standard deviation of the background fluorescence intensity, which is measured between cycle 3 and 15 (Fig 1A). The $\log_{10}$ of number of targets initially present is proportional with the C_t and can be measured on a standard curve (12).

In order to confirm the specifity of the TaqMan PCR assay, several herpesvirus strains were tested. No cross-reactivity was observed between HCMV and herpes simplex virus types 1 and 2, Epstein–Barr virus or human herpesviruses 6 and 8 (data not shown). In addition, a BLAST search performed to check the specificity of the DNA sequences of the HCMV primer and probe sets showed no genomic cross-reactivity with other viruses or cells.

Assay sensitivity and range of detection of the TaqMan PCR assay were determined by testing the ready standards in the range 10^7–10 copies per ml. Figure 1(A) shows representative amplification plots of the TaqMan assay. When plotting C_ts against $\log_{10}$ of the HCMV copy number per reaction, linearity was observed over the range of 10^7–10 copy reactions (Fig 1B).

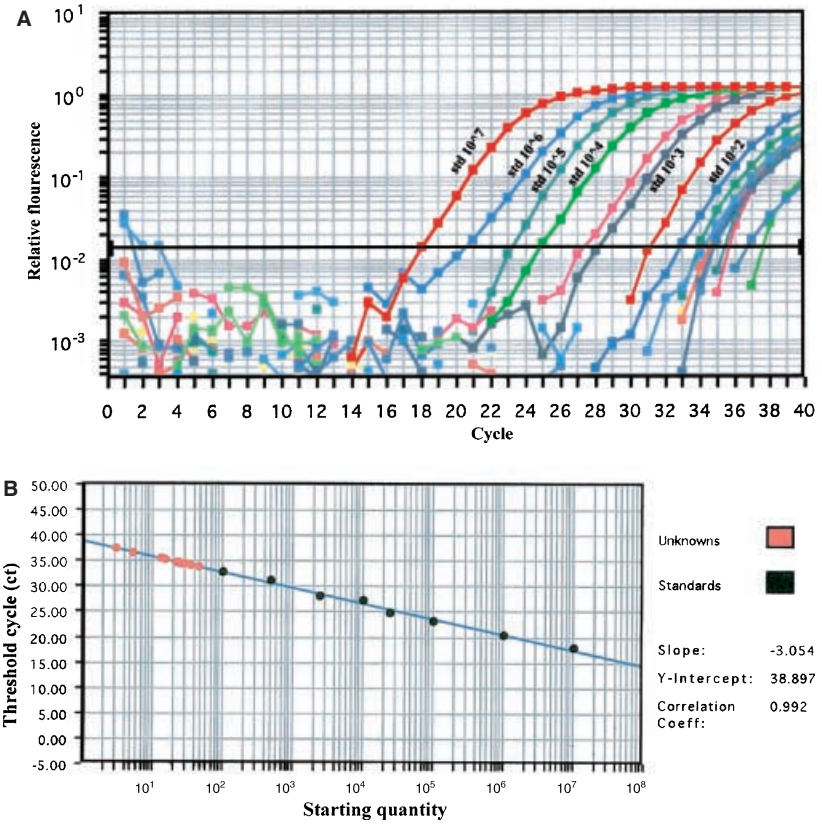

Fig. 1. Amplification of human cytomegalovirus (HCMV) DNA. Serial dilutions of the HCMV standard were used as template for real-time polymerase chain reaction (PCR). The amount of HCMV DNA in each sample is shown as an amplification plot. The amplification plot reflects the generation of the reporter dye during amplification and is directly related to the formation of PCR products. Intersection between the amplification plot and the threshold (horizontal line) is defined as the cycle threshold (C_t) value. The C_t value is related directly to the amount of PCR product and therefore related to the original amount of target present in the PCR reaction. A standard curve was produced from the amplification plot in panel A (correlation coefficient = 0.992). As described above, threshold cycle is the cycle number at which the threshold fluorescence is reached.

Statistical analysis

Statistical analyses were carried out using the chi-squared test, Fisher exact test and Mann–Whitney *U*-test (SPSS 10.0 statistical package; SPSS Inc, Chicago, IL, USA). *p*-values equal to or less than 0.05 were considered statistically significant.

Results

Table 1 describes clinical characteristics of the study subjects having aggressive periodontitis or periodontal health. Except for age, the means of all clinical variables were significantly different between the two subject groups.

A total of 11 of the 16 (68.8%) patients with aggressive periodontitis revealed HCMV in the subgingival specimen. None of the 15 healthy control subjects yielded HCMV. In aggressive periodontitis patients, periodontal sites with positive HCMV identification vs. no HCMV identification were associated with increased pocket depth and probing attachment loss but did not differ significantly in Plaque Index or Gingival Index (Table 2).

Table 3 shows that the HCMV-positive subgingival specimens averaged between 5×10^2 and 7.4×10^3

Table 1. Clinical description of study subjects with aggressive periodontitis and healthy periodontium

Items	Aggressive periodontitis ($n = 16$)	Periodontal health ($n = 15$)	*p*-values
Age in years	24.1 ± 3.4[a]	23.7 ± 2.9	Non-significant
Plaque Index, whole mouth	1.9 ± 0.5	0.7 ± 0.3	< 0.0001
Gingival Index, whole mouth	1.9 ± 0.1	0.4 ± 0.4	< 0.0001
Pocket depth in mm at sampling sites	7.7 ± 1.5	2.1 ± 0.6	< 0.0001
Probing attachment loss in mm at sampling sites	7.8 ± 1.6	2.1 ± 0.6	< 0.0001

[a]Mean ± standard deviation.

Table 2. Clinical variables for HCMV-positive and HCMV-negative aggressive periodontitis lesions

Items	HCMV-positive sites ($n = 11$)	HCMV-negative sites ($n = 5$)	*p*-values
Plaque Index at sampling sites	1.8 ± 0.4[a]	2.1 ± 0.5	Non-significant
Gingival Index at sampling sites	1.9 ± 0.1	2.0 ± 0.10	Non-significant
Whole mouth pocket depth in mm	5.2 ± 1.0	4.5 ± 0.8	Non-significant
Pocket depth in mm at sampling sites	8.3 ± 1.3	6.4 ± 0.9	0.01
Whole mouth probing attachment loss in mm	5.2 ± 1.2	4.4 ± 0.6	Non-significant
Probing attachment loss in mm at sampling sites	8.4 ± 1.4	6.4 ± 0.9	0.04

[a]Mean ± standard deviation.
HCMV, human cytomegalovirus.

Table 3. Subgingival HCMV copies in infected aggressive periodontitis lesions

Patient no.	Mean periodontal pocket depth in mm	Mean HCMV-counts/ml subgingival suspension
1	10	6.8×10^3
2	10	2.8×10^3
3	7	3.0×10^3
4	8	5.0×10^3
5	8	5.0×10^2
6	9	2.0×10^3
7	7	5.0×10^3
8	8	3.4×10^3
9	7	7.4×10^3
10	10	2.4×10^3
11	7	5.2×10^3
Mean	**8.3**	$\mathbf{4.0 \times 10^3}$

HCMV, human cytomegalovirus.

viral copies/ml, corrected for sample dilution. No relationship was found between mean periodontal pocket depth and HCMV subgingival count.

Discussion

Recent studies have pointed to the feasibility of using TaqMan real-time PCR assay for quantification of cytomegalovirus DNA in biological specimens (18, 19). Our study suggests that the TaqMan technology also represents a valuable method to determine HCMV levels in periodontitis sites.

A real-time fluorescence-based PCR assay has several advantages over gel-based PCR assays.

(i) It does not rely on the final product of PCR amplification for quantification and therefore overcomes the problem that small variations in amplification efficiency early in the thermocycling process can give rise to large variations in the final quantity of amplified products.
(ii) It has high specificity due to the binding of two primers and one probe and, if the probe binds non-specifically to sequences other than the target sequence, it will not be cleaved or detected as part of the amplification.
(iii) It allows a wide dynamic range of detection since the measured DNA copy number is directly proportional to the initial copy number.
(iv) It operates in a closed system, avoiding contamination.
(v) It lends itself to large-scale analysis due to the availability of high-throughput sequence detection systems.
(vi) Results can be obtained within a few hours.

A quantitative PCR assay will have application in periodontology as a research tool and probably also in supporting diagnostic tests. Quantitative PCR assays may help determine if a certain threshold number of periodontal HCMV is predictive of periodontal disease progression, and may provide the opportunity to monitor treatment efficacy, in particular the use of chemotherapeutics affecting HCMV.

The present findings provide further compelling evidence that HCMV participates in the pathogenesis of aggressive periodontitis. We detected HCMV in 68.8% of subgingival specimens from aggressive periodontitis lesions and did not find the virus in healthy periodontal sites. Kamma *et al*. (7) identified HCMV in 59.4% of aggressive periodontitis lesions that

have revealed progression within the preceding 6 months. The present study found deeper probing pocket depth and more attachment loss in HCMV-positive than in HCMV-negative periodontal sites. Ting *et al*. (3) also detected HCMV more frequently in deep than in shallow periodontal sites of localized aggressive periodontitis patients. Contreras & Slots (20) identified HCMV with higher frequency in deep than in shallow periodontal pockets of adult patients. The association of HCMV with deepening probing depths is consistent with a periodontopathic role of the virus. Moreover, we found no statistically significant difference in Gingival Index between HCMV-positive and HCMV-negative study sites. Kamma *et al*. (7) also showed that HCMV single infection occurred with similar frequency in bleeding and non-bleeding periodontal sites of aggressive periodontitis patients. That HCMV seems to be more strongly associated with periodontitis than with gingivitis provides additional evidence for a role of the virus in destructive periodontal disease.

The real-time PCR technique used in the present study revealed HCMV loads ranging from 5×10^2 to 7.4×10^3 copies/ml in the 11 viral-positive specimens studied. Although the HCMV load may be argued to be relatively low, because of its absence in healthy periodontal sites, the virus may anyway be important in the etiopathogenesis of aggressive periodontitis. The method of sample collection is probably an important determinant of the quantity of HCMV recovered. Contreras *et al*. (21) showed that inflamed gingival tissue contains higher level of herpesviruses than the associated periodontal pocket. HCMV infects mainly periodontal macrophages and lymphocytes, which predominate in gingival tissue, and may only sporadically infect polymorphonuclear leukocytes, which predominate in periodontal pockets (22). Since our study examined pocket samples, it may have underestimated the amount of HCMV present in tissue in close proximity to the site of active periodontal breakdown. Moreover, it is likely that a single stoke by a curette only collects a relatively small portion of the total subgingival deposit present. On the other hand, the use of a curette for subgingival sampling may yield more material than can be obtained by using the conventional paper point method of sampling (23, 24).

The absence of HCMV in some periodontitis lesions is also of interest. Negative HCMV DNA results may in part be due to sampling of stable periodontal sites. Kamma *et al*. (7) detected HCMV, EBV-1, HSV and herpesvirus co-infection in progressing periodontal sites with significantly higher frequency than in stable sites. Also, Contreras & Slots (25) reported a positive relationship between herpesvirus periodontal infection and the clinical severity of periodontal disease. However, the present study did not assess periodontal disease activity. Further studies are needed to compare the quantity of HCMV in periodontitis active and stable sites. Such studies may form the basis for establishing a critical threshold value for HCMV that would identify an increased risk for periodontal attachment loss.

In conclusion, the present data indicate that the TaqMan quantitative HCMV assay represents an attractive method for measuring HCMV viral load in periodontal sites. Quantification of HCMV may provide valuable information for understanding the role of the virus in periodontal pathosis and should prove useful for monitoring the antiviral efficacy of periodontal treatment. Further studies are also warranted to determine levels of HCMV in types of periodontal disease other than aggressive periodontitis.

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